DNA encoding human α-and β-subunits of neuronal nicotinic acetylcholine receptor, cells transformed therewith, and a recombinant cell line expressing a functional human $\alpha_7$ subunit of neuronal nicotinic acetylcholine receptor

This application is a continuation-in-part of U.S. application Serial No. 08/487,596 filed 6/7/95, now pending, which is a continuation-in-part of U.S. application Serial No. 08/149,503, filed November 8, 1993, now pending; and a continuation-in-part of U.S. application Serial No. 08/028,031, filed March 8, 1993, now abandoned; and a continuation-in-part of U.S. application Serial No. 07/938,154, filed November 30, 1992, which is a continuation-in-part of U.S. application Serial No. 07/504,455, filed April 3, 1990, now issued as U.S. Letters Patenet No. 5,369,028, each of which is hereby incorpoarted by referenece herein in their entirety.

This invention relates to DNA encoding human neuronal nicotinic acetylcholine receptor protein subunits, as well as the proteins themselves. In particular, human neuronal nicotinic acetylcholine receptor α-subunit-encoding DNA, α-subunit proteins, β-subunit-encoding DNA, β-subunit proteins, and combinations thereof are provided. A non-human cell line expressing a human α-subunit protein is also disclosed.

## BACKGROUND OF THE INVENTION

Ligand-gated ion channels provide a means for communication between cells of the central nervous system. These channels convert a signal (e.g., a chemical referred to as a neurotransmitter) that is released by one cell into an electrical signal that propagates along a target cell membrane. A variety of neurotransmitters and neurotransmitter receptors exist in the central and peripheral nervous systems. Five families of ligand-gated receptors, including the nicotinic acetylcholine receptors (NAChRs) of neuromuscular and neuronal origins, have been identified (Stroud et al. (1990) Biochemistry 29:11009-11023). There is, however, little understanding of the manner in which the variety of receptors generates different responses to neurotransmitters or to other modulating ligands in different regions of the nervous system.

The nicotinic acetylcholine receptors (NAChRs) are multisubunit proteins of neuromuscular and neuronal origins. These receptors form ligand-gated ion channels that mediate synaptic transmission between nerve and muscle and between neurons upon interaction with the neurotransmitter acetylcholine (ACh). Since various nicotinic acetylcholine receptor (NAChR) subunits exist, a variety of NAChR compositions (i.e., combinations of subunits) exist. The different NAChR compositions exhibit different specificities for various ligands and are thereby pharmacologically distinguishable. Thus, the nicotinic acetylcholine receptors expressed at the vertebrate neuromuscular junction in vertebrate sympathetic ganglia and in the vertebrate central nervous system have been distinguished on the basis of the effects of various ligands that bind to different NAChR compositions. For example, the elapid α-neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction do not block activation of some neuronal nicotinic acetylcholine receptors that are expressed on several different neuron-derived cell lines.

Muscle NAChR is a glycoprotein composed of five subunits with the stoichiometry $\alpha_2\beta(\gamma$ or $\epsilon)\delta$. Each of the subunits has a mass of about 50-60 kilodaltons (kd) and is encoded by a different gene. The $\alpha_2\beta(\gamma$ or $\epsilon)\delta$ complex forms functional receptors containing two ligand binding sites and a ligand-gated transmembrane channel. Upon interaction with a cholinergic agonist, muscle nicotinic AChRs conduct sodium ions. The influx of sodium ions rapidly short-circuits the normal ionic gradient maintained across the plasma membrane, thereby depolarizing the membrane. By reducing the potential difference across the membrane, a chemical signal is transduced into an electrical signal that signals muscle contraction at the neuromuscular junction.

Functional muscle nicotinic acetylcholine receptors have been formed with $\alpha\beta\delta\gamma$ subunits, $\alpha\beta\gamma$ subunits, $\alpha\beta\delta$ subunits, $\beta\delta\gamma$ subunits or $\alpha\delta$ subunits, but not with only one subunit (see e.g., Kurosaki et al. (1987) FEBS Lett. 214:253-258; Camacho et al. (1993) J. Neuroscience 13:605-613). In contrast, functional neuronal AChRs (nAChRs) can be formed from $\alpha$ subunits alone or combinations of $\alpha$ and $\beta$ subunits. The larger $\alpha$ subunit is generally believed to be the ACh-binding subunit and the lower molecular weight $\beta$ subunit is generally believed to be the structural subunit, although it has not been definitively demonstrated that the $\beta$ subunit does not have the ability to bind ACh. Each of the subunits which participate in the formation of a functional ion channel are, to the extent they contribute to the structure of the resulting channel, "structural" subunits, regardless of their ability (or inability) to bind ACh. Neuronal AChRs (nAChRs), which are also ligand-gated ion channels, are expressed in ganglia of the autonomic nervous system and in the central nervous system (where they mediate signal transmission), in post-synaptic locations (where they modulate transmission), and in pre- and extra-synaptic locations (where they may have additional functions).

DNA encoding NAChRs has been isolated from several sources. Based on the information available from such work, it has been evident for some time that NAChRs expressed in muscle, in autonomic ganglia, and in the central nervous system are functionally diverse. This functional diversity could be due, at least in part, to the large number of different NAChR subunits which exist. There is an incomplete understanding, however, of how (and which) NAChR subunits combine to generate unique NAChR subtypes, particularly in neuronal cells. Indeed, there is evidence that only certain NAChR subtypes may be involved in diseases such as Alzheimer's disease. Moreover, it is not clear whether NAChRs from analogous tissues or cell types are similar across species.

Accordingly, there is a need for the isolation and characterization of DNAs encoding each human neuronal NAChR subunit, recombinant cells containing such subunits and receptors prepared therefrom. In order to study the function of human neuronal AChRs and to obtain disease-specific pharmacologically active agents, there is also a need to obtain isolated (preferably purified) human neuronal nicotinic AChRs, and isolated (preferably purified) human neuronal nicotinic AChR subunits. In addition, there is also a need to develop assays to identify such pharmacologically active agents.

The availability of such DNAs, cells, receptor subunits and receptor compositions will eliminate the uncertainty of speculating as to human nNAChR structure and function based on predictions drawn from non-human nNAChR data, or human or non-human muscle or ganglia NAChR data.

Therefore, it is an object herein to isolate and characterize DNA encoding subunits of human neuronal nicotinic acetylcholine receptors. It is also an object herein to provide methods for recombinant production of human neuronal nicotinic acetylcholine receptor subunits. It is also an object herein to provide purified receptor subunits and to provide methods for screening compounds to identify compounds that modulate the activity of human neuronal AChRs.

Likewise, it is an object of the present invention to provide a recombinant non-human cell line transformed with a heterologous nucleic acid molecule that encodes a human $\alpha$ subunit of neuronal nAChR.

These and other objects will become apparent to those of skill in the art upon further study of the specification and claims.

## BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated DNAs encoding novel human alpha and beta subunits of neuronal NAChRs. Also provided is a non-human cell line that expresses a human $\alpha_7$ subunit of neuronal nAChR. In particular, isolated DNA encoding human $\alpha_4$, $\alpha_7$, and $\beta_4$ subunits of neuronal NAChRs are provided. Messenger RNA and polypeptides encoded by the above-described DNA are also provided.

Further in accordance with the present invention, there are provided recombinant human neuronal nicotinic AChR subunits, including $\alpha_4$, $\alpha_7$, and $\beta_4$ subunits, as well as methods for the production thereof. In addition, recombinant human neuronal nicotinic acetylcholine receptors containing at least one human neuronal nicotinic AChR subunit are also provided, as well as methods for the production thereof. Further provided are recombinant neuronal nicotinic AChRs that contain a mixture of one or more NAChR subunits encoded by a host cell, and one or more nNAChR subunits encoded by heterologous DNA or RNA (i.e., DNA or RNA as described herein that has been introduced into the host cell), as well as methods for the production thereof.

Plasmids containing DNA encoding the above-described subunits are also provided. Recombinant cells containing the above-described DNA, mRNA or plasmids are also provided herein. Such cells are useful, for example, for replicating DNA, for producing human NAChR subunits and recombinant receptors, and for producing cells that express receptors containing one or more human subunits.

Also provided in accordance with the present invention are methods for identifying cells that express functional nicotinic acetylcholine receptors. Methods for identifying compounds which modulate the activity of NAChRs are also provided. Invention methods employ that isolated DNAs, encoding human $\alpha$ and $\beta$ subunits of neuronal AChRs and polypeptides encoded thereby.

The DNA, mRNA, vectors, receptor subunits, receptor subunit combinations and cells provided herein permit production of selected neuronal nicotinic AChR receptor subtypes and specific combinations thereof, as well as antibodies to said receptor subunits. This provides a means to prepare synthetic or recombinant receptors and receptor subunits that are substantially free of

contamination from many other receptor proteins whose presence can interfere with analysis of a single NAChR subtype. The availability of desired receptor subtypes makes it possible to observe the effect of a drug substance on a particular receptor subtype and to thereby perform initial in vitro screening of the drug substance in a test system that is specific for humans and specific for a human neuronal nicotinic AChR subtype.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed for diagnostic and therapeutic applications.

The ability to screen drug substances in vitro to determine the effect of the drug on specific receptor compositions should permit the development and screening of receptor subtype-specific or disease-specific drugs. Also, testing of single receptor subunits or specific receptor subtype combinations with a variety of potential agonists or antagonists provides additional information with respect to the function and activity of the individual subunits and should lead to the identification and design of compounds that are capable of very specific interaction with one or more of the receptor subunits or receptor subtypes. The resulting drugs should exhibit fewer unwanted side effects than drugs identified by screening with cells that express a variety of subtypes.

Further in relation to drug development and therapeutic treatment of various disease states, the availability of DNAs encoding human nNAChR subunits enables identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA sequences which can then be introduced into laboratory animals or in vitro assay systems to determine the effects thereof.

## DETAILED DESCRIPTION OF THE FIGURES

FIG. 1 presents a restriction map of two pCMV promoter-based vectors, pCMV-T7-2 and pCMV-T7-3.

FIG. 2 presents a restriction map of a pCMV promoter based vector, pcDNA3-KE$\alpha_7$RBS.

FIG. 3 depicts the nicotine and acetylcholine –induced dose-response curves foe the A7 cell line obtained from functional bulk calcium assays.

FIG. 4 depicts the kinetics of the A7 stable cell line obatined by electrophysiological analysis.

FIG. 5 depicts the MLA and $\alpha$-bungarotoxin (ligands of A7) binding assay of A7.

FIG. 6 depicts the results of a single cell calcium imaging of the A7 cell line, showing the homogeneity of the response of the A7 cell line to acetylcholine.

FIG. 7 depicts the results of a Western blot analysis using an A-7 specific antibody as a probe. The data specifically confirm expression of the $\alpha_7$ protein by the A7 cells.

FIG. 8 shows the results of a Northen Blot analysis of total RNA prepared from A7 cells.

FIG. 9 a - b compares agonist–induced dose-response curves of the alpha3beta2alpha5 expressing cells and alpha3beta2 expressing cells and specifically shows that their profile differs from that of an alpha3beta2 subunit combination.

FIG. 10 depicts a comparison in the kinetics of decay of currents induced by acetylcholine between A3B2A5 cells and A3B2 cells.

FIG. 11 confirms the association of the alpha3 and beta 2 with alpha 5 subunits in cell line A3B2A5.

FIG. 12 depicts the expression construct for alpha 3 - -pc DNA3-KEalpha3

FIG. 13 depicts the expression construct for alpha 5 - -pHook3-KEalpha5RBS

FIG. 14 depicts the expression construct for beta 2-- pc DNA3-KEbeta2RBS

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have isolated and characterized DNAs encoding novel human alpha and beta subunits of neuronal nAChR. Specifically, isolated DNAs encoding human $\alpha_4$, $\alpha_7$, and $\beta_4$ subunits of neuronal Anchors are described herein. Recombinant messenger RNA (mRNA) and recombinant polypeptides encoded by the above-described DNA are also provided.

In accordance with the present invention, we have developed methods for identifying compounds that modulate the activity of nAChRs, which employ DNAs encoding human $\alpha$ and $\beta$ subunits of neuronal nAChRs and polypeptides encoded thereby. Specifically, screening methods employing DNAs encoding human $\alpha_2$, $\alpha_3$,$\alpha_4$, $\alpha_5$, $\alpha_6$, $\alpha_7$, $\beta_2$, $\beta_3$, $\beta_4$, subunits of neuronal NAChRs is described herein.

Also described are isolated cells experiencing various multimeric combinations of human $\alpha$ and $\beta$ subunits of neuronal nAChRs, i.e., 3-, 4- and 5-way combinations. A non-human cell line expressing human $\alpha_7$ subunit is also described herein.

As used herein, isolated (or substantially pure) as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their in vivo cellular environments through the efforts of human beings. Thus as used herein, isolated (or substantially pure) DNA refers to DNAs purified according to standard techniques employed by those skilled in the art (see, e.g., Maniatis et al. (1982) *Molecular*

*Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Similarly, as used herein, "recombinant" as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been prepared by the efforts of human beings, e.g., by cloning, recombinant expression, and the like. Thus as used herein, recombinant proteins, for example, refers to proteins produced by a recombinant host, expressing DNAs which have been added to that host through the efforts of human beings.

As used herein, a human alpha subunit gene is a gene that encodes an alpha subunit of a human neuronal nicotinic acetylcholine receptor. The alpha subunit is a subunit of the nAChR to which ACh binds. Assignment of the name "alpha" to a putative nNAChR subunit, according to Deneris et al. [Tips (1991) 12:34-40] is based on the conservation of adjacent cysteine residues in the presumed extracellular domain of the subunit that are the homologues of cysteines 192 and 193 of the Torpedo alpha subunit (see Noda et al. (1982) Nature 299:793-797). As used herein, an alpha subunit subtype refers to a human nNAChR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nNAChR alpha subunit-encoding DNAs (or deposited clones) disclosed herein. An alpha subunit also binds to ACh under physiological conditions and at physiological concentrations and, in the optional presence of a beta subunit (i.e., some alpha subunits are functional alone, while others require the presence of a beta subunit), generally forms a functional AChR as assessed by methods described herein or known to those of skill in this art.

Also contemplated are alpha subunits encoded by DNAs that encode alpha subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under specified hybridization conditions. Such subunits also form a functional receptor, as assessed by the methods described herein or known to those of skill in the art, generally with one or more beta subunit subtypes. Typically, unless an alpha subunit is encoded by RNA that arises from alternative splicing (i.e., a splice variant), alpha-encoding DNA and the alpha subunit encoded thereby share substantial sequence homology with at least one of the alpha subunit DNAs (and proteins encoded thereby) described or deposited herein. It is understood that DNA or RNA encoding a splice variant may overall share less than 90% homology with the DNA or RNA provided herein, but include regions of nearly 100% homology to a DNA fragment or deposited clone described herein, and encode an open reading frame that includes start and stop codons and encodes a functional alpha subunit.

As used herein, a splice variant refers to variant NAChR subunit-encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic DNA, resulting in the production of more than one type of mRNA. cDNA derived from differentially processed genomic DNA will encode NAChR subunits that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic sequence can lead to the production of multiple, related mRNAs and proteins. Both the resulting mRNAs and proteins are referred to herein as "splice variants".

Stringency of hybridization is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ($T_m$) of the hybrids. $T_m$ can be approximated by the formula:

$81.5° C.-16.6(\log_{10} [Na^+])+0.41(\%G+C)-600/l$,

where l is the length of the hybrids in nucleotides. $T_m$ decreases approximately 1°-1.5° C. with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein:

(1) HIGH STRINGENCY refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65° C. (i.e., if a hybrid is not stable in 0.018M NaCl at 65° C., it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.2% SDS at 42° C., followed by washing in 0.1× SSPE, and 0.1% SDS at 65° C.;

(2) MODERATE STRINGENCY refers to conditions equivalent to hybridization in 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.2% SDS at 42° C., followed by washing in 0.2× SSPE, 0.2% SDS, at 65° C.; and

(3) LOW STRINGENCY refers to conditions equivalent to hybridization in 10% formamide, 5× Denhardt's solution, 6× SSPE, 0.2% SDS, followed by washing in 1× SSPE, 0.2% SDS, at 50° C.

It is understood that these conditions may be duplicated using a variety of buffers and temperatures and that they are not necessarily precise.

Denhardt's solution and SSPE (see, e.g., Sambrook, Fritsch, and Maniatis, in: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers. For example, SSPE is pH 7.4 phosphate-buffered 0.18M NaCl. SSPE can be prepared, for example, as a 20× stock solution by dissolving 175.3 g of NaCl, 27.6 g of $NaH_2 PO_4$ and 7.4 g EDTA in 800 ml of water, adjusting the pH to 7.4, and then adding water to 1 liter. Denhardt's solution (see, Denhardt (1966) Biochem. Biophys. Res. Commun. 23:641) can be prepared, for example, as a 50× stock solution by mixing 5 g Ficoll (Type 400, Pharmacia LKB Biotechnology, INC., Piscataway N.J.), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V; Sigma, St. Louis Mo.) water to 500 ml and filtering to remove particulate matter.

The phrase "substantial sequence homology" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species having substantial sequence homology are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard,

"slight and non-consequential sequence variations" mean that "homologous" sequences, i.e., sequences that have substantial homology with the DNA, RNA, or proteins disclosed and claimed herein, are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

In practice, the term substantially the same sequence means that DNA or RNA encoding two proteins hybridize under conditions of high stringency and encode proteins that have the same sequence of amino acids or have changes in sequence that do not alter their structure or function. As used herein, substantially identical sequences of nucleotides share at least about 90% identity, and substantially identical amino acid sequences share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

As used herein, "$\alpha_2$ subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:1, or to the DNA of deposited clone having ATCC Accession No. 68277, or to DNA that encodes the amino acid sequence set forth in SEQ.ID.No:2. Typically, unless an $\alpha_2$ subunit arises as a splice variant, an $\alpha_2$ DNA shares substantial sequence homology (greater than about 90%) with the $\alpha_2$ DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, "$\alpha_3$ subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:3, or to the DNA of deposited clone having ATCC Accession No. 68278, or to DNA that encodes the amino acid sequence set forth in SEQ.ID.No:4 Typically, unless an $\alpha_3$ arises as a splice variant, an $\alpha_3$ DNA shares substantial sequence homology (greater than about 90%) with the $\alpha_3$ DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above described DNA.

As used herein, "$\alpha_5$ subunit DNA" refers to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, as described, for example, by Chini et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:1572-1576.

The phrase "substantially the same" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have

slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are substantially the same are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that sequences that are substantially the same as the DNA, RNA, or proteins disclosed and claimed herein are functionally equivalent to the human-derived sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the human-derived nucleic acid and amino acid composotions disclosed and claimed herein. In particular, functionally equivalent DNAs encode human-derived proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution on a non-polar residue for another non-polar residue or a charged residue for a similarly chared residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

As used herein, "$\alpha_4$ subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example

said DNA may encode the amino acid sequence set forth in SEQ.ID.No:6, or

said DNA may encode the amino acid sequence encoded by clone HnAChR$\alpha$4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may encode the amino acid sequence encoded by clone HnAChR$\alpha$4.1, deposited under ATCC Accession No. 69152.

Presently preferred $\alpha_4$-encoding DNAs can be characterized as follows

said DNA may hybridize to the coding sequence set forth in SEQ.ID.No:5 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 184-2067) under high stringency conditions, or

said DNA may hybridize under high stringency conditions to the sequence (preferably to substantially the entire sequence) of the $\alpha_4$-encoding insert of clone HnAChR$\alpha$4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA may hybridize under high stringency conditions to the sequence of the $\alpha_4$-encoding insert of clone HnAChR$\alpha$4.1, deposited under ATCC Accession No. 69152.

Especially preferred $\alpha_4$-encoding DNAs of the invention are characterized as follows

DNA having substantially the same nucleotide sequence as the coding region set forth in SEQ.ID.No:5 (i.e., nucleotides 184-2067 thereof), or

DNA having substantially the same nucleotide sequence as the $\alpha_4$-encoding insert of clone HnAChR$\alpha$4.2, deposited under ATCC Accession No. 69239, or

the 5' nucleotides of said DNA have substantially the same sequence as the $\alpha_4$-encoding insert of clone HnAChR$\alpha$4.1, deposited under ATCC Accession No. 69152.

Typically, unless an $\alpha_4$ subunit arises as a splice variant, $\alpha_4$-encoding DNA will share substantial sequence homology (i.e., greater than about 90%), with the $\alpha_4$ DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNAs.

As used herein, "$\alpha_3$ subunit DNA" referes to DNA that encodes a human neuronal nicotinic acetylcholine receptor subunit of the same name, and to DNA that hyridizes under conditions of high stringency to the DNA of SEQ ID No. 7, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 8. Typically, unles an $\alpha_5$ subunit arises as a splice variant, an $\alpha_5$ DNA shares substantial sequence homology (greater than about 90%) with the $\alpha_5$ DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA described herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA. Human $\alpha$5 subunit DNA has been described in the art, for example, by Chini et al. (1992) Proc. Natl. Acad. Sci. USA 89: 1572-1576.

As used herein, "$\alpha_6$ subunit DNA" refers to DNA that encodes a neuronal subunit of the same name, and to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 9, or to DNA that encodes the amino acid sequence set forth in SEQ ID No. 10. Typically, unless and $\alpha_6$ arises as a splice variant, an $\alpha_6$ DNA shares substantial sequence homology (greater than about 90%) with the $\alpha_6$ DNA described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of neraly 100% homology to the above described DNA.

As used herein, "$\alpha_7$ subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ.ID.No:8. Presently preferred $\alpha_7$-encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ.ID.No:7 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 73-1581). Especially preferred $\alpha_7$-encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as the coding sequence set forth in SEQ.ID.No:7 (i.e., nucleotides 73-1581 thereof).

Typically, unless an $\alpha_7$ subunit arises as a splice variant, $\alpha_7$-encoding DNA will share substantial sequence homology (greater than about 90%) with the $\alpha_7$ DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.

The $\alpha_7$ subunits derived from the above-described DNA are expected to bind to the neurotoxin α-bungarotoxin α-bgtx). The activity of AChRs that contain α7 subunits should be inhibited upon interaction with α-bgtx. Amino acid residues 210 through 217, as set forth in SEQ.ID.No:8, are believed to be important elements in the binding of α-bgtx (see, for example, Chargeux et al. Trends Pharmacol Sci. (1992) 13:299-301).

As used herein, a human beta subunit gene is a gene that encodes a beta subunit of a human neuronal nicotinic acetylcholine receptor. Assignment of the name "beta" to a putative nNAChR subunit, according to Deneris et al. supra, is based on the lack of adjacent cysteine residues (which are characteristic of alpha subunits). The beta subunit is frequently referred to as the structural NAChR subunit (although it is possible that beta subunits also have ACh binding properties). Combination of beta subunit(s) with appropriate alpha subunit(s) leads to the formation of a functional receptor. As used herein, a beta subunit subtype refers to a nNAChR subunit that is encoded by DNA that hybridizes under high stringency conditions to at least one of the nNAChR-encoding DNAs (or deposited clones) disclosed herein. A beta subunit forms a functional NAChR, as assessed by methods described herein or known to those of skill in this art, with appropriate alpha subunit subtype(s).

Also contemplated are beta subunits encoded by DNAs that encode beta subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under the specified hybridization conditions. Such subunits also form functional receptors, as assessed by the methods described herein or known to those of skill in the art, in combination with appropriate alpha subunit subtype(s). Typically, unless a beta subunit is encoded by RNA that arises as a splice variant, beta-encoding DNA and the beta subunit encoded thereby share substantial sequence homology with the beta-encoding DNA and beta subunit protein described herein. It is understood that DNA or RNA encoding a splice variant may share less than 90% overall homology with the DNA or RNA provided herein, but such DNA will include regions of nearly 100% homology to the DNA described herein.

As used herein, "$\beta_2$ subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 13, or to the DNA of deposited clone HnAChRβ2, having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 14. Typically, unless a $\beta_2$ subunit arises as a splice variant, a $\beta_2$ DNA shares substantial sequence homology (greater than about 90%) with the $\beta_2$ DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, "$\beta_3$ subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID No. 15, or to DNA encoding the amino acid sequence set forth in SEQ ID No. 16. Typically, unless a $\beta_3$ subunit arises as a splice variant, a $\beta_2$ DNA shares substantial sequence homology (greater than about 90%) with the $\beta_3$ DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but

such a splice variant would include regions of nearly 100% homology to the above-described DNA.

As used herein, "$\beta_4$ subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ.ID.No:18. Presently preferred $\beta_4$-encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ.ID.No:17 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 87-1583). Especially preferred $\beta_4$-encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as set forth in SEQ.ID.No:17.

Typically, unless a $\beta_4$ subunit arises as a splice variant, $\beta_4$-encoding DNA will share substantial sequence homology (greater than about 90%) with the $\beta_4$ DNAs described or deposited herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such DNA would include regions of nearly 100% homology to the above-described DNA.

DNA encoding human neuronal nicotinic AChR alpha and beta subunits may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with DNA disclosed herein (including nucleotides derived from any of SEQ.ID.Nos:1, 3, 5, 7, 9, 11, 13, 15 or 17, or with any of the deposited clones referred to herein. Suitable libraries can be prepared from neuronal tissue samples, hippocampus tissue, or cell lines, such as the human neuroblastoma cell line IMR32 (ATCC Accession No. CCL127), and the like. The library is preferably screened with a portion of DNA including the entire subunit-encoding sequence thereof, or the library may be screened with a suitable probe.

As used herein, a probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 bases set forth in any of SEQ.ID.Nos:1, 3, 5, 7, 9, or 11, or in the subunit encoding DNA in any of the deposited clones described herein (e.g., ATCC accession no. 69239 or 69152). Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode the cytoplasmic loop, signal sequences, acetylcholine (ACh) and $\alpha$-bungarotoxin ($\alpha$-bgtx) binding sites, and the like. Amino acids 210-220 are typically involved in ACh and $\alpha$-bgtx binding. The approximate amino acid residues which comprise such regions for other preferred probes are set forth in the following table:

| Subunit | Signal Sequence | TMD1* | TMD2 | TMD3 | TMD4 | Cytoplasmic Loop |
|---|---|---|---|---|---|---|
| $\alpha_2$ | 1-55 | 264-289 | 297-320 | 326-350 | 444-515 | 351-443 |
| $\alpha_3$ | 1-30 | 240-265 | 273-296 | 302-326 | 459-480 | 327-458 |
| $\alpha_4$ | 1-33 | 241-269 | 275-289 | 303-330 | 593-618 | 594-617 |

| | | | | | | |
|---|---|---|---|---|---|---|
| $\alpha_5$ | 1-22 | 250-275 | 282-304 | 310-335 | 422-437 | 336-421 |
| $\alpha_6$ | 1-30 | 240-265 | 272-294 | 301-326 | 458-483 | 327-457 |
| $\alpha_7$ | 1-23 | 229-256 | 262-284 | 290-317 | 462-487 | 318-461 |
| $\beta_2$ | 1-25 | 234-259 | 267-288 | 295-320 | 453-477 | 321-452 |
| $\beta_3$ | 1-20 | 232-258 | 265-287 | 293-318 | 421-446 | 319-420 |
| $\beta_4$ | 1-23 | 234-258 | 264-285 | 290-319 | 454-478 | 320-453 |

*TMD = transmembrane domain

Alternatively, portions of the DNA can be used as primers to amplify selected fragments in a particular library.

After screening the library, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein or with the deposited clones described herein, to ascertain whether they include DNA encoding a complete alpha or beta subunit. If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If desired, the library can be rescreened with positive clones until overlapping clones that encode an entire alpha or beta subunit are obtained. If the library is a cDNA library, then the overlapping clones will include an open reading frame. If the library is genomic, then the overlapping clones may include exons and introns. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

Complementary DNA clones encoding various subtypes of human nNAChR alpha and beta subunits have been isolated. Each subtype of the subunit appears to be encoded by a different gene. The DNA clones provided herein may be used to isolate genomic clones encoding each subtype and to isolate any splice variants by screening libraries prepared from different neural tissues. Nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of human NAChR subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns that correspond to different splice variants of transcripts encoding human NAChR subunits.

It has been found that not all subunit subtypes are expressed in all neural tissues or in all portions of the brain. Thus, in order to isolate cDNA encoding particular subunit subtypes or splice variants of such subtypes, it is preferable to screen libraries prepared from different neuronal or neural tissues. Preferred libraries for obtaining DNA encoding each subunit include: hippocampus to isolate human $\alpha_4$– and $\alpha_5$-encoding DNA; IMR32 to isolate human $\alpha_3$-, $\alpha_5$-, $\alpha_7$- and $\beta_4$-encoding DNA, thalamus to isolate $\alpha_2$ and $\beta_2$-encoding DNA; and the like.

It appears that the distribution of expression of human neuronal nicotinic AChRs differs from the distribution of such receptors in rat. For example, RNA encoding the rat $\alpha_4$ subunit is abundant

in rat thalamus, but is not abundant in rat hippocampus (see, e.g., Wada et al. (1989) J. Comp. Neurol 284:314-335). No $\alpha_4$-encoding clones could be obtained, however, from a human thalamus library. Instead, human $\alpha_4$ clones were ultimately obtained from a human hippocampus library. Thus, the distribution of $\alpha_4$ nNAChR subunit in humans and rats appears to be quite different.

Rat $\alpha_3$ subunit appears to be a CNS-associated subunit that is abundantly expressed in the thalamus and weakly expressed in the brain stem (see, e.g., Boulter et al. (1986) Nature 319:368-374; Boulter et al. (1987) Proc. Natl. Acad. Sci. USA 84:7763-7767; and Wada et al. (1989) J. Comp. Neurol 284:314-335). In efforts to clone DNA encoding the human nicotinic AChR $\alpha_3$ subunit, however, several human libraries, including a thalamus library, were unsuccessfully screened. Surprisingly, clones encoding human $\alpha_3$ subunit were ultimately obtained from a brain stem library and from IMR32 cells that reportedly express few, if any, functional nicotinic acetylcholine receptors (see, e.g., Gotti et al. ((1986) Biochem. Biophys. Res. Commun. 137:1141-1147, and Clementi et al. (1986) J. Neurochem. 47:291-297).

Rat $\alpha_7$ subunit transcript reportedly is abundantly expressed in the hippocampus (see Seguela et al. (1993) J. Neurosci. 13:596-604). Efforts to clone DNA encoding a human $\alpha_7$ subunit from a human hippocampus library ($1\times10^6$ recombinants) were unsuccessful. Surprisingly, clones encoding a human NAChR $\alpha_7$ subunit were ultimately obtained from an IMR32 cell cDNA library.

The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the level of skill of the art.

An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of affecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently preferred plasmids for expression of invention AChR subunits in eukaryotic host cells, particularly mammalian cells, include SV40 promoter-based expression vectors, such as pZeoSV (Invitrogen, San Diego, CA) CMV; cytomegalovirus (CMV) promoter-based vectors such as, pcDNA1, pcDNA3, pCEP4, (Invitrogen, San Diego, CA); and MMTV promoter-based vector such as pMAMneo (Clentech, Inc.) and the like.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors.

Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

As used herein, the term "operatively linked" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove or alter 5' untranslated portions of the clones to remove extra, potential alternative translation initiation (i.e., start) codons or other sequences that interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, for example, Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon to enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCDNA1 (Invitrogen, San Diego, Calif.), and MMTV promoter-based vectors such as pMSG (Catalog No. 27-4506-01 from Pharmacia, Piscataway, N.J.).

Full-length DNAs encoding human neuronal NAChR subunits have been inserted into vector pCMV-T7, a pUC19-based mammalian cell expression vector containing the CMV promoter/enhancer, SV40 splice/donor sites located immediately downstream of the promoter, a polylinker downstream of the splice/donor sites, followed by an SV40 polyadenylation signal. Placement of NAChR subunit DNA between the CMV promoter and SV40 polyadenylation signal provides for constitutive expression of the foreign DNA in a mammalian host cell transfected with the construct. For inducible expression of human NAChR subunit-encoding DNA in a mammalian cell, the DNA can be inserted into a plasmid such as PMSG. This plasmid contains the mouse mammary tumor virus (MMTV) promoter for steroid- inducible expression of operatively associated foreign DNA. If the host cell does not express endogenous glucocorticoid receptors required for uptake of glucocorticoids (i.e., inducers of the MMTV promoter) into the cell, it is necessary to additionally transfect the cell with DNA encoding the glucocorticoid receptor (ATCC accession no. 67200). Full-length human DNA clones encoding

human $\alpha_3$, $\alpha_4$, $\alpha_7$, $\beta_2$ and $\beta_4$ have also been subcloned into pIBI24 (International Biotechnologies, Inc., New Haven, Conn.) or pCMV-T7-2 for synthesis of *in vitro* transcripts.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described polynucleic acids (i.e., DNA or mRNA). Such host cells as bacterial, yeast and mammalian cells can be used for replicating DNA and producing nAChR subunit(s). Methods for constructing expression vectors, preparing in vitro transcripts, transfecting DNA into mammalian cells, injecting oocytes, and performing electrophysiological and other analyses for assessing receptor expression and function as described herein are also described in PCT Application Nos. PCT/US91/02311, PCT/US91/05625 and PCT/US92/11090, and in co-pending U.S. application Ser. Nos. 07/504,455, 07/563,751 and 07/812,254. The subject matter of these applications are hereby incorporated by reference herein in their entirety.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous DNA by $CaPO_4$ precipitation (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. 76:1373-1376). Recombinant cells can then be cultured under conditions whereby the subunit(s) encoded by the DNA is (are) expressed. Preferred cells include mammalian cells (e.g., HEK 293, CHO and $Ltk^-$ cells), yeast cells (e.g., methylotrophic yeast cells, such as *Pichia pastoris*), bacterial cells (e.g., *Escherichia coli*), and the like.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells (such as, for example, *P. pastoris* (see U.S. Pat. Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), *Saccharomyces cerevisiae, Candida tropicalis, Hansenula polymorpha*, and the like), mammalian expression systems, including commercially available systems and other such systems known to those of skill in the art, for expression of DNA encoding the human neuronal nicotinic AChR subunits provided herein are presently preferred. Xenopus oocytes are preferred for expression of RNA transcripts of the DNA.

In preferred embodiments, DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express a specific human nNAChR receptor subtype, or specific combinations of subtypes. The resulting cell lines can then be produced in quantity for reproducible quantitative analysis of the effects of drugs on receptor function. In other embodiments, mRNA may be produced by in vitro transcription of DNA encoding each subunit. This mRNA, either from a single subunit clone or from a combination of clones, can then be injected into Xenopus oocytes where the RNA directs the synthesis of the human receptor subunits, which then form functional receptors. Alternatively, the subunit-encoding DNA can be directly injected into oocytes for expression of functional receptors. The transfected mammalian cells or injected oocytes may then be used in the methods of drug screening provided herein.

Cloned full-length DNA encoding any of the subunits of human neuronal nicotinic AChR may be introduced into a plasmid vector for expression in a eukaryotic cell. Such DNA may be

genomic DNA or cDNA. Host cells may be transfected with one or a combination of plasmids, each of which encodes at least one human neuronal nicotinic AChR subunit.

Eukaryotic cells in which DNA or RNA may be introduced include any cells that are transfectable by such DNA or RNA or into which such DNA or RNA may be injected. Preferred cells are those that can be transiently or stably transfected and also express the DNA and RNA. Presently most preferred cells are those that can form recombinant or heterologous human neuronal nicotinic AChRs comprising one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected.

Exemplary cells for introducing DNA include cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae, Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK 293 (which are available from ATCC under accession #CRL 1573; Ltk$^-$ cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhrf$^-$ CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12:555). Presently preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension and that can be frozen in liquid nitrogen and then thawed and regrown. HEK 293 cells are described, for example, in U.S. Pat. No. 5,024,939 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol. 5:2051-2060).

DNA may be stably incorporated into cells or may be transiently introduced using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected cells under conditions selective for cells expressing the marker gene. To produce such cells, the cells should be transfected with a sufficient concentration of subunit-encoding nucleic acids to form human neuronal nicotinic AChRs that contain the human subunits encoded by heterologous DNA. The precise amounts and ratios of DNA encoding the subunits may be empirically determined and optimized for a particular combination of subunits, cells and assay conditions. Recombinant cells that express neuronal nicotinic AChR containing subunits encoded only by the heterologous DNA or RNA are especially preferred.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Similarly, the human neuronal nicotinic AChR subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that specifically bind to one or more of the subunits may be used for affinity purification of the subunit or human neuronal nicotinic AChRs containing the subunits.

In accordance with one embodiment of the present invention, methods for producing cells that express human neuronal nicotinic AChR subunits and functional receptors are also provided. In one such method, host cells are transfected with DNA encoding at least one alpha subunit of a neuronal nicotinic acetylcholine receptor and at least one beta subunit of a neuronal nicotinic acetylcholine receptor. Using methods such as northern blot or slot blot analysis, transfected cells that contain alpha and/or beta subunit encoding DNA or RNA can be selected. Transfected cells are also analyzed to identify those that express NAChR protein. Analysis can be carried out, for example, by measuring the ability of cells to bind acetylcholine, nicotine, or a nicotine agonist, compared to the nicotine binding ability of untransfected host cells or other suitable control cells, by electrophysiologically monitoring the currents through the cell membrane in response to a nicotine agonist, and the like.

In particularly preferred aspects, eukaryotic cells which contain heterologous DNAs express such DNA and form recombinant functional neuronal nicotinic AChR(s). In more preferred aspects, recombinant neuronal nicotinic AChR activity is readily detectable because it is a type that is absent from the untransfected host cell or is of a magnitude not exhibited in the untransfected cell. Such cells that contain recombinant receptors could be prepared, for example, by causing cells transformed with DNA encoding the human neuronal nicotinic AChR $\alpha_3$ and $\beta_4$ subunits to express the corresponding proteins. The resulting synthetic or recombinant receptor would contain only the $\alpha_3$ and $\beta_4$ nNAChR subunits. Such a receptor would be useful for a variety of applications, e.g., as part of an assay system free of the interferences frequently present in prior art assay systems employing non-human receptors or human tissue preparations. Furthermore, testing of single receptor subunits with a variety of potential agonists or antagonists would provide additional information with respect to the function and activity of the individual subunits. Such information may lead to the identification of compounds which are capable of very specific interaction with one or more of the receptor subunits. Such specificity may prove of great value in medical application.

Thus, DNA encoding one or more human neuronal nicotinic AChR subunits may be introduced into suitable host cells (e.g., eukaryotic or prokaryotic cells) for expression of individual subunits and functional NAChRs. Preferably combinations of alpha and beta subunits may be introduced into cells: such combinations include combinations of any one or more of al, $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$ and $\alpha_7$ with $\beta_2$ or $\beta_4$. Sequence information for $\alpha_1$ is presented in Biochem. Soc. Trans. (1989) 17:219-220; sequence information for $\alpha_5$ is presented in Proc. Natl. Acad. Sci. USA (1992) 89:1572-1576; and sequence information for $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_7$, $\beta_2$ and $\beta_4$ is presented in the Sequence Listing provided herewith. Presently preferred combinations of subunits include any one or more of $\alpha_1$, $\alpha_2$, $\alpha_3$ or $\alpha_5$ with $\beta_4$; or $\alpha_4$ or $\alpha_7$ in combination with either $\beta_2$ or $\beta_4$. It is recognized that some of the subunits may have ion transport function in the absence of additional subunits. For example, the $\alpha_7$ subunit is functional in the absence of any added beta subunit.

In acordance with the above, also disclosed are cells transfected or transformed with DNA or RNA encoding multimeric human NAChR subunit combinations. These include but are not limited to the following:

Multimeric Subunit Combinations

α2β4α6
α3β4α6
α4β4α5
α4β4α6
α4β2α5
α4β2β3
α3β2α6β3
α2β4α5
α2β2α5
α3β2α5
α3β4α5

Also contemplated are cells expressing one or more α subunit with more than one β subunit. These include but are not limited to the following subunit combinations:
αXβ2β4 (where X defines one or more of the alpha sununits disclosed herein)
αXβ2β3β4
α2β2α6
α3β2α6
α4β2α6
aXb2b3 (where X defines one or more of the alpha subunits disclosed herein)

Stable cell lines expressing any of the above referenced multimeric subunit combinations are also a feature of the invention.

As used herein, "$\beta_2$ subunit DNA" refers to DNA that encodes a neuronal subunit of the same name and, to DNA that hybridizes under conditions of high stringency to the DNA of SEQ.ID.No:9, or to the DNA of deposited clone HnAChβ62, having ATCC Accession No. 68279, or to DNA encoding the amino acid sequence set forth in SEQ.ID.No:10. Typically, unless a $\beta_2$ subunit arises as a splice variant, a $\beta_2$ DNA shares substantial sequence homology (greater than about 90%) with the $\beta_2$ DNA described herein. DNA or RNA encoding a splice variant may share overall less than 90% homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNA.

In certain embodiments, eukaryotic cells with heterologous human neuronal nicotinic AChRs are produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human neuronal nicotinic AChR. In preferred embodiments, the subunits that are translated include an alpha subunit of a human neuronal nicotinic AChR. More preferably, the composition that is introduced contains an RNA transcript which encodes an alpha subunit and also contains an RNA transcript which encodes a beta subunit of a human neuronal nicotinic AChR. RNA transcripts can be obtained from cells transfected with DNAs encoding human neuronal nicotinic acetylcholine receptor subunits or by in vitro transcription of subunit-encoding DNAs. Methods for in vitro transcription of cloned DNA and injection of the resulting mRNA into eukaryotic cells are well known in the art.

Amphibian oocytes are particularly preferred for expression of in vitro transcripts of the human nNAChR DNA clones provided herein. See, for example, Dascal (1989) CRC Crit. Rev. Biochem. 22:317-387, for a review of the use of Xenopus oocytes to study ion channels.

Thus, pairwise (or stepwise) introduction of DNA or RNA encoding alpha and beta subtypes into cells is possible. The resulting cells may be tested by the methods provided herein or known to those of skill in the art to detect functional AChR activity. Such testing will allow the identification of pairs of alpha and beta subunit subtypes that produce functional AChRs, as well as individual subunits that produce functional AChRs.

An alternative embodiment is drawn to a non-human cell line that stably expresses the $\alpha_7$ nAChR. Preferably, the non-human cell line expressing the human $\alpha_7$ nAChR subunit is a rat cell line, i.e., the $GH_4C_1$ cell line.

As used herein, $GH_4C_1$ cells are derived from rat pituitary tumor tissue and are transfected with DNA or RNA encoding the human $\alpha_7$ nAChR.

As used herein, activity of a human neuronal nicotinic AChR refers to any activity characteristic of an NAChR. Such activity can typically be measured by one or more in vitro methods, and frequently corresponds to an in vivo activity of a human neuronal nicotinic AChR. Such activity may be measured by any method known to those of skill in the art, such as, for example, measuring the amount of current which flows through the recombinant channel in response to a stimulus.

Methods to determine the presence and/or activity of human neuronal nicotinic AChRs include assays that measure nicotine binding, $^{86}Rb$ ion-flux, $Ca^{2+}$ influx, the electrophysiological response of cells, the electrophysiological response of oocytes transfected with RNA from the cells, and the like. In particular, methods are provided herein for the measurement or detection of an AChR-mediated response upon contact of cells containing the DNA or mRNA with a test compound.

As used herein, a recombinant or heterologous human neuronal nicotinic AChR refers to a receptor that contains one or more subunits encoded by heterologous DNA that has been introduced into and expressed in cells capable of expressing receptor protein. A recombinant human neuronal nicotinic AChR may also include subunits that are produced by DNA endogenous to the host cell. In certain embodiments, recombinant or heterologous human neuronal nicotinic AChR may contain only subunits that are encoded by heterologous DNA.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome of the cell in which it is present or to DNA or RNA which is found in a location or locations in the genome that differ from that in which it occurs in nature. Typically, heterologous or foreign DNA and RNA refers to DNA or RNA that is not endogenous to the host cell and has been artificially introduced into the cell. Examples of heterologous DNA include DNA that encodes a human neuronal nicotinic AChR subunit, DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes, and the

like. The cell that expresses heterologous DNA may contain DNA encoding the same or different expression products. Heterologous DNA need not be expressed and may be integrated into the host cell genome or maintained episomally.

Recombinant receptors on recombinant eukaryotic cell surfaces may contain one or more subunits encoded by the DNA or mRNA encoding human neuronal nicotinic AChR subunits, or may contain a mixture of subunits encoded by the host cell and subunits encoded by heterologous DNA or mRNA. Recombinant receptors may be homogeneous or may be a mixture of subtypes. Mixtures of DNA or mRNA encoding receptors from various species, such as rats and humans, may also be introduced into the cells. Thus, a cell can be prepared that expresses recombinant receptors containing only $\alpha_3$ and $\beta_4$ subunits, or any other combination of alpha and beta subunits provided herein. For example, $\alpha_4$ and/or $\alpha_7$ subunits of the present invention can be co-expressed with $\beta_2$ and/or $\beta_4$ receptor subunits; similarly, $\beta_4$ subunits according to the present invention can be co-expressed with $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$ and/or $\alpha_7$ receptor subunits. As noted previously, some of the nNAChR subunits may be capable of forming functional receptors in the absence of other subunits, thus co-expression is not always required to produce functional receptors.

As used herein, a functional neuronal nicotinic AChR is a receptor that exhibits an activity of neuronal nicotinic AChRs as assessed by any in vitro or in vivo assay disclosed herein or known to those of skill in the art. Possession of any such activity that may be assessed by any method known to those of skill in the art and provided herein is sufficient to designate a receptor as functional. Methods for detecting NAChR protein and/or activity include, for example, assays that measure nicotine binding, $^{86}Rb$ ion-flux, $Ca^{2+}$ influx, the electrophysiological response of cells containing heterologous DNA or mRNA encoding one or more receptor subunit subtypes, and the like. Since all combinations of alpha and beta subunits may not form functional receptors, numerous combinations of alpha and beta subunits should be tested in order to fully characterize a particular subunit and cells which produce same. Thus, as used herein, "functional" with respect to a recombinant or heterologous human neuronal nicotinic AChR means that the receptor channel is able to provide for and regulate entry of human neuronal nicotinic AChR-permeable ions, such as, for example, $Na^+$, $K^+$, $Ca^{2+}$ or $Ba^{2+}$, in response to a stimulus and/or bind ligands with affinity for the receptor. Preferably such human neuronal nicotinic AChR activity is distinguishable, such as by electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous nicotinic AChR activity that may be produced by the host cell.

In accordance with a particular embodiment of the present invention, recombinant human neuronal nicotinic AChR-expressing mammalian cells or oocytes can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the AChR-mediated response in the presence and absence of test compound, or by comparing the AChR-mediated response of test cells, or control cells (i.e., cells that do not express nNAChRs), to the presence of the compound.

As used herein, a compound or signal that "modulates the activity of a neuronal nicotinic AChR" refers to a compound or signal that alters the activity of NAChR so that activity of the NAChR is different in the presence of the compound or signal than in the absence of the compound or

signal. In particular, such compounds or signals include agonists and antagonists. The term agonist refers to a substance or signal, such as ACh, that activates receptor function; and the term antagonist refers to a substance that interferes with receptor function. Typically, the effect of an antagonist is observed as a blocking of activation by an agonist. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for the agonist (e.g., ligand or neurotransmitter) for the same or closely situated site. A non-competitive antagonist or blocker inactivates the functioning of the receptor by interacting with a site other than the site that interacts with the agonist.

As understood by those of skill in the art, assay methods for identifying compounds that modulate human neuronal nicotinic AChR activity (e.g., agonists and antagonists) generally require comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound, except the control culture is not exposed to test compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence and absence of test compound, by merely changing the external solution bathing the cell. Another type of "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells, except the cells employed for the control culture do not express functional human neuronal nicotinic AChRs. In this situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound being assayed.

The functional recombinant human neuronal nicotinic AChR includes at least an alpha subunit, or an alpha subunit and a beta subunit of a human neuronal nicotinic AChR. Eukaryotic cells expressing these subunits have been prepared by injection of RNA transcripts and by transfection of DNA. Such cells have exhibited nicotinic AChR activity attributable to human neuronal nicotinic AChRs that contain one or more of the heterologous human neuronal nicotinic AChR subunits. For example, *Xenopus laevis* oocytes that had been injected with in vitro transcripts of the DNA encoding human neuronal nicotinic AChR $\alpha_3$ and $\beta_4$ subunits exhibited AChR agonist induced currents; whereas cells that had been injected with transcripts of either the $\alpha_3$ or $\beta_4$ subunit alone did not. In addition, HEK 293 cells that had been co-transfected with DNA encoding human neuronal NAChR $\alpha_3$ and $\beta_4$ subunits exhibited AChR agonist-induced increases in intracellular calcium concentration, whereas control HEK 293 cells (i.e., cells that had not been transfected with $\alpha_3$– and $\beta_4$-encoding DNA) did not exhibit any AChR agonist-induced increases in intracellular calcium concentration.

With respect to measurement of the activity of functional heterologous human neuronal nicotinic AChRs, endogenous AChR activity and, if desired, activity of AChRs that contain a mixture of endogenous host cell subunits and heterologous subunits, should, if possible, be inhibited to a significant extent by chemical, pharmacological and electrophysiological means.

Deposits

The deposited clones have been deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., U.S.A. 20852, under the terms of the Budapest Treaty on

the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. Patent based on this or any application claiming priority to or incorporating this application by reference thereto, all restrictions upon availability of the deposited material will be irrevocably removed.

The invention will now be described in greater detail with reference to the following non-limiting examples.

EXAMPLE 1

Isolation of DNA Encoding Human nNAChR Subunits

A. DNA Encoding a Human nNAChR $\beta_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from the IMR32 human neuroblastoma cell line (the cells had been treated with dibutyryl cAMP and bromodeoxyuridine prior to constructing the library). The library constructed from the cDNAs was screened with a fragment of a rat nicotinic AChR $\beta_4$ subunit cDNA. Hybridization was performed at 42° C. in 5× SSPE, 5× Denhardt's solution, 50% formamide, 200 $\mu$g/ml herring sperm DNA and 0.2% SDS. Washes were performed in 0.1× SSPE, 0.2% SDS at 65° C. Five clones were identified that hybridized to the probe.

The five clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. The insert DNA of one of the five clones contained the complete coding sequence of a $\beta_4$ subunit of a human nicotinic AChR (see nucleotides 87-1583 of SEQ.ID.No:11). The amino acid sequence deduced from the nucleotide sequence of the full-length clone has ~81% identity with the amino acid sequence deduced from the rat nicotinic AChR $\beta_4$ subunit DNA. Several regions of the deduced rat and human $\beta_4$ amino acid sequences are notably dissimilar: amino acids 1-23 (the human sequence has only ~36% identity with respect to the rat sequence), 352-416 (the human sequence has only ~48% identity with respect to the rat sequence), and 417-492 (the human sequence has only ~78% identity with respect to the rat sequence). Furthermore, amino acids 376-379 in the rat $\beta_4$ subunit are not contained in the human $\beta_4$ subunit.

B. DNA Encoding a Human nNAChR $\alpha_7$ Subunit

An amplified IMR32 cell cDNA library ($1\times10^6$ recombinants; cells treated with dibutyryl cAMP and bromodeoxyuridine) was screened with a fragment of a rat nicotinic AChR $\alpha_7$ subunit cDNA. The hybridization conditions were identical to those described above for screening an IMR32 cell cDNA library with the rat $\beta_4$ subunit DNA. Washes were performed in 0.2× SSPE,

0.2% SDS at 65° C. Seven positive clones were identified by hybridization to the labeled rat DNA probe. Six of the clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. One of the clones contains the complete coding sequence of a human AChR receptor $\alpha_7$ subunit gene (see nucleotides 73-1581 of SEQ.ID.No:7).

C. DNA Encoding a Human nNAChR $\alpha_4$ Subunit

Random primers were used in synthesizing cDNA from RNA isolated from human hippocampus tissue. cDNAs larger than 2.0 kb were inserted into the λgt10 phage vector to create a cDNA library. Approximately $1\times10^6$ recombinants were screened with a fragment of a DNA encoding a rat nicotinic AChR $\alpha_4$ subunit using the same hybridization and washing conditions as described above for screening an IMR32 cell cDNA library for $\alpha_7$ subunit cDNAs. Three clones hybridized strongly to the probe. Two of these three clones, designated KEα4.1 and KEα4.2, have been deposited with the American Type Culture Collection (ATCC, Rockville, Md.) and assigned accession nos. 69152 and 69239, respectively.

Characterization of the plaque-purified clones revealed that one of the clones, KEα4.2, contains the complete coding sequence of a human nicotinic AChR α4 subunit gene (coding sequence of this human $\alpha_4$ subunit cDNA is provided as nucleotides 184-2067 in SEQ.ID.No:5). Comparison of the 5' ends of the coding sequences of the human and rat α4 subunit cDNAs reveals that the rat sequence contains an 18-nucleotide segment that is not present in the human sequence.

D. DNA Encoding Human nNAChR $\alpha_2$, $\alpha_3$, & $\beta_2$ Subunits

Plasmids containing DNA that encodes and/or that can be used to isolate DNA that encodes human neuronal nicotinic acetylcholine receptor $\alpha_2$, $\alpha_3$ and $\beta_2$ subunits have been deposited with the American Type Culture Collection (ATCC). The clone names and deposit accession numbers are:

| Subunit | Clone Name | ATCC Accession No. |
|---|---|---|
| $\alpha_2$ | HnAChRα2 | 68277 |
| $\alpha_3$ | HnACHRα3 | 68278 |
| $\beta_2$ | HnAChRβ2 | 68279 |

In addition, DNA sequences that encode full-length $\alpha_2$, $\alpha_3$ and $\beta_2$ subunits are set forth in SEQ.ID.Nos:1, 3 and 9, respectively.

EXAMPLE 2

I. Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic AChR Subunits

Isolated cDNAs encoding human neuronal nicotinic AChR subunits were incorporated into vectors for use in expressing the subunits in mammalian host cells and for use in generating in vitro transcripts of the DNAs to be expressed in Xenopus oocytes. Several different vectors were utilized in preparing the constructs as follows.

A. Construct for Expression of a Human nNAChR $\alpha_3$ Subunit

DNA encoding a human neuronal nicotinic AChR $\alpha_3$ subunit was subcloned into the pCMV-T7-2 general expression vector to create pCMV-KE$\alpha$3. Plasmid pCMV-T7-2 (see FIG. 1) is a pUC19-based vector that contains a CMV promoter/enhancer, SV40 splice donor/splice acceptor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the SV40 splice sites, an SV40 polyadenylation signal downstream of the T7 promoter, and a polylinker between the T7 promoter and the polyadenylation signal. This vector thus contains all the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 promoter is located just upstream of the polylinker, this plasmid can be used for synthesis of in vitro transcripts of heterologous DNA that has been subcloned into the vector at the polylinker. FIG. 1 also shows a restriction map of pCMV-T7-3. This plasmid is identical to pCMV-T7-2 except that the restriction sites in the polylinker are in the opposite order as compared to the order in which they occur in pCMV-T7-2.

A 1.7 kb SfiI (blunt-ended)/EcoRI DNA fragment containing nucleotides 27-1759 of SEQ.ID.No:3 (i.e., the entire $\alpha_3$ subunit coding sequence plus 12 nucleotides of 5' untranslated sequence and 206 nucleotides of 3' untranslated sequence) was ligated to EcoRV/EcoRI-digested pCMV-T7-2 to generate pCMV-KE$\alpha$3. Plasmid pCMV-KE$\alpha$3 was used for expression of the $\alpha_3$ subunit in mammalian cells and for generating in vitro transcripts of the $\alpha_3$ subunit DNA.

B. Constructs for Expression of a Human nNAChR $\beta_4$ Subunit

A 1.9 kb EcoRI DNA fragment containing nucleotides 1-1915 of SEQ. ID.No:11 (i.e., the entire $\beta_4$ subunit coding sequence plus 86 nucleotides of 5' untranslated sequence and 332 nucleotides of 3' untranslated sequence) was ligated to EcoRI-digested pGEM7Zf(+) (Promega Catalog #P2251; Madison, Wis.). The resulting construct, KE$\beta$4.6/pGEM, contains the T7 bacteriophage RNA polymerase promoter in operative association with two tandem $\beta_4$ subunit DNA inserts (in the same orientation) and was used in generating in vitro transcripts of the DNA.

The same 1.9 kb EcoRI DNA fragment containing nucleotides 1-1915 of SEQ.ID.No:11 was ligated as a single insert to EcoRI-digested pCMV-T7-3 to generate pCMV-KE$\beta$4. Plasmid pCMV-KE$\beta$4 was used for expression of the $\beta_4$ subunit in mammalian cells and for generating in vitro transcripts of the $\beta_4$ subunit DNA.

C. Constructs for Expression of a Human nNAChR $\alpha_7$ Subunit

Two pCMV-T7-2-based constructs were prepared for use in recombinant expression of a human neuronal nicotinic AChR $\alpha_7$ subunit. The first construct, pCMV-KE$\alpha$7.3, was prepared by ligating a 1.9 kb XhoI DNA fragment containing nucleotides 1-1876 of SEQ. ID.No:7 (i.e., the entire $\alpha_7$ subunit coding sequence plus 72 nucleotides of 5' untranslated sequence and 295 nucleotides of 3' untranslated sequence) to SalI-digested pCMV-T7-3. The second construct, pCMV-KE$\alpha$7, was prepared by replacing the 5' untranslated sequence of the 1.9 kb XhoI $\alpha_7$ subunit DNA fragment described above with a consensus ribosome binding site (5'-GCCACC-3'; see Kozak (1987) Nucl. Acids Res. 15:8125-8148). The resulting modified fragment was ligated as a 1.8 kb BglII/XhoI fragment with BglII/SalI-digested pCMV-T7-2 to generate pCMV-KE$\alpha$7. Thus, in pCMV-KE$\alpha$7, the translation initiation codon of the coding sequence of the $\alpha_7$ subunit cDNA is preceded immediately by a consensus ribosome binding site.

D. Constructs for Expression of a Human nNAChR $\beta_2$ Subunit

DNA fragments encoding portions of a human neuronal nicotinic AChR $\beta_2$ subunit were ligated together to generate a full-length $\beta_2$ subunit coding sequence contained in plasmid pIBI24 (International Biotechnologies, Inc. (IBI), New Haven, Conn.). The resulting construct, H$\beta$2.1F, contains nucleotides 1-2450 of SEQ.ID.No:9 (i.e., the entire $\beta_2$ subunit coding sequence, plus 266 nucleotides of 5' untranslated sequence and 675 nucleotides of 3' untranslated sequence) in operative association with the T7 promoter. Therefore, H$\beta$2.1F was used for synthesis of in vitro transcripts from the $\beta_2$ subunit DNA..

Since the 5' untranslated sequence of the $\beta_2$ subunit DNA contains a potential alternative translation initiation codon (ATG) beginning 11 nucleotides upstream (nucleotides 256-258 in SEQ.ID.No:9) of the correct translation initiation codon (nucleotides 267-269 in SEQ. ID.No:9), and because the use of the upstream ATG sequence to initiate translation of the $\beta_2$ DNA would result in the generation of an inoperative peptide (because the upstream ATG is not in the correct reading frame), an additional $\beta_2$-encoding construct was prepared as follows. A 2.2 kb KspI/EcoRI DNA fragment containing nucleotides 262-2450 of SEQ.ID.No:9 was ligated to pCMV-T7-2 in operative association with the T7 promoter of the plasmid to create pCMV-KE$\beta$2. The $\beta_2$ subunit DNA contained in pCMV-KE$\beta$2 retains only 5 nucleotides of 5' untranslated sequence upstream of the correct translation initiation codon.

EXAMPLE 3

Expression of Recombinant Human Nicotinic AChR in Oocytes

Xenopus oocytes were injected with in vitro transcripts prepared from constructs containing DNA encoding $\alpha_3$, $\alpha_7$, $\beta_2$ and $\beta_4$ subunits. Electrophysiological measurements of the oocyte transmembrane currents were made using the two-electrode voltage clamp technique (see, e.g., Stuhmer (1992) *Meth. Enzymol.* 207:319-339).

1. Preparation of *in vitro* transcripts

Recombinant capped transcripts of pCMV-KE$\alpha$3, pCMV-KE$\beta$2, KE$\beta$4.6/pGEM and pCMV-KE$\beta$4 were synthesized from linearized plasmids using the mCAP RNA Capping Kit (Cat. #200350 from Stratagene, Inc., La Jolla, Calif.). Recombinant capped transcripts of pCMV-KE$\alpha$7, pCMV-KE$\alpha$7.3 and H$\beta$2.1F were synthesized from linearized plasmids using the MEGAscript T7 in vitro transcription kit according to the capped transcript protocol provided by the manufacturer (Catalog #1334 from AMBION, Inc., Austin, Tex.). The mass of each synthesized transcript was determined by UV absorbance and the integrity of each transcript was determined by electrophoresis through an agarose gel.

2. Electrophysiology

Xenopus oocytes were injected with either 12.5, 50 or 125 ng of human nicotinic AChR subunit transcript per oocyte. The preparation and injection of oocytes were carried out as described by Dascal (1987) in *Crit. Rev. Biochem.* 22:317-387. Two-to-six days following mRNA injection, the oocytes were examined using the two-electrode voltage clamp technique. The cells were bathed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3) containing 1 $\mu$M atropine with or without 100 $\mu$M d-tubocurarine. Cells were voltage-clamped at -60 to -80 mV. Data were acquired with Axotape software at 2-5 Hz. The agonists acetylcholine (ACh), nicotine, and cytisine were added at concentrations ranging from 0.1 $\mu$M to 100 $\mu$M. The results of electrophysiological analyses of the oocytes are summarized in Table 1.

TABLE 1

| Template, ng RNA Injected | Number of oocytes responding | Current Agonists | Amplitude |
|---|---|---|---|
| pCMV-KEα3, 12.5 ng | 0 of 8 | ACh, Nicotine | |
| KEβ4.6/pGEM, 12.5 ng | 0 of 9 | ACh, Nicotine | |
| pCMV-KEα3, 12.5 ng + KEβ4.6/pGEM, 12.5 ng | 4 of 5 | ACh, Nicotine | 20-550 nA |
| pCMV-KEα3, 12.5 ng + KEβ4.6/pGEM, 12.5 ng | 3 of 4 | ACh, Cytisine, Nicotine | 20-300 nA |
| pCMV-KEα3, 125 ng + and pCMV-KEβ4, 125 ng | 5 of 5 | Ch, Nicotine, Cytisine | 200-500 nA |
| pCMV-KEα3, 125 ng + pCMV-KEβ4, 125 ng | 6 of 6 | ACh, Nicotine, Cytisine | 100-400 nA |
| pCMV-KEα7.3, 125 ng | 3 of 15 | Ach | ~20 nA |
| pCMV-KEα7, 125 ng | 11 of 11 | Ach | 20-250 nA |
| pCMV-KEα3, 12.5 ng + pCMV-KEβ2, 12.5 ng | 2 of 9 | ACh, Nicotine | <10 nA |
| pCMV-KEα3, 125 ng + pCMV-KEβ2, 125 ng | 0 of 9 | ACh, Nicotine | |
| pCMV-KEα3, 125 ng + Hβ2.1 F, 125 ng | 13 of 16 | Ach (100 $\mu$M) ACh (300 $\mu$M) | ~20 nA ~80 nA |

a. Oocytes Injected with $\alpha_3$ and/or $\beta_4$ Transcripts

Oocytes that had been injected with 12.5 ng of the $\alpha_3$ transcript or 12.5 ng of the $\beta_4$ transcript did not respond to application of up to 100 $\mu$M ACh, nicotine or cytisine. Thus, it appears that these subunits do not form functional homomeric nicotinic AChR channels. By contrast, oocytes injected with 12.5 or 125 ng of the $\alpha_3$ transcript and 12.5 ng or 125 ng of the $\beta_4$ transcript exhibited detectable inward currents in response to ACh, nicotine, and cytisine at the tested concentrations (0.1 $\mu$M to 10 $\mu$M). Some differences in the kinetics of the responses to cytisine compared to nicotine and ACh were observed. The relative potency of the agonists appeared to be cytisine>ACh>nicotine, which differs from the results of similar studies of oocytes injected with transcripts of the rat nicotinic AChR $\alpha_3$ and $\beta_4$ subunits (see, for example, Luetje et al. (1991) *J. Neurosci.* 11:837-845).

The responses to ACh and nicotine were reproducibly blocked by d-tubocurarine. For example, complete blockage of the response to ACh was observed in the presence of 100 $\mu$M d-tubocurarine. The inhibition appeared to be reversible. The responses to ACh, nicotine and cytisine were also at least partially blocked by 100 nM mecamylamine.

The current response of $\alpha_3$-$\beta_4$-injected oocytes to 10 $\mu$M ACh was also examined in terms of membrane voltage. In these experiments, voltage steps were applied to the cells in the presence of ACh. The graph of current vs. voltage appeared typical of responses observed for $Na^+$, $K^+$ - permeable channels. For example, the zero current level (reversal potential) is less than -40 mV. The contribution of $Ca^{++}$ flux to the total current can be ascertained by varying the calcium concentration in the external medium and taking multiple current measurements at different holding potentials around the reversal potential. Such studies indicate that the channel carrying the current generated in response to ACh treatment of $\alpha_3$-$\beta_4$-injected oocytes is permeable to $Na^+$, $K^+$ and $Ca^{++}$.

b. Oocytes injected with $\alpha_7$ subunit transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR $\alpha_7$ subunit. Plasmid pCMV-KE$\alpha$7.3 contains the $\alpha_7$ subunit coding sequence with 72 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE$\alpha$7 contains the $\alpha_7$ subunit coding sequence devoid of any 5' untranslated sequence and further contains a consensus ribosome binding site immediately upstream of the coding sequence.

Oocytes injected with 125 ng of $\alpha_7$ transcript synthesized from pCMV-KE$\alpha$7 displayed inward currents in response to 10 or 100 $\mu$M ACh. This response was blocked by 100 $\mu$M d-tubocurarine.

Oocytes injected with 125 ng of $\alpha_7$ transcript synthesized from pCMV-KE$\alpha$7.3 exhibited ACh-induced currents that were substantially weaker than those of oocytes injected with $\alpha_7$ transcript synthesized from pCMV-KE$\alpha$7. These results indicate that human neuronal nicotinic AChR $\alpha_7$

subunit transcripts generated from $\alpha_7$ subunit DNA containing a ribosome binding site in place of 5' untranslated sequence may be preferable for expression of the $\alpha_7$ receptor in oocytes.

c. Oocytes injected with $\alpha_3$ and $\beta_2$ subunit transcripts

As described in Example 2, two constructs were prepared for use in expressing the human neuronal nicotinic AChR $\beta_2$ subunit. Plasmid H$\beta$2.1F contains the $\beta_2$ subunit coding sequence with 266 nucleotides of 5' untranslated sequence upstream of the translation initiation codon. Plasmid pCMV-KE$\beta$2 contains the $\beta_2$ subunit coding sequence and only 5 nucleotides of 5' untranslated sequence upstream of the translation initiation codon.

Oocytes injected with transcripts of pCMV-KE$\alpha$3 and pCMV-KE$\beta$2 displayed no current in response to nicotinic AChR $\alpha_3$ agonists. In contrast, oocytes injected with transcripts of pCMV-KE$\alpha$3 and H$\beta$2.1F displayed ~20 nA inward currents in response to 100 $\mu$M ACh and ~80 nA inward currents in response to 300 $\mu$M ACh. The current response was blocked by 100 $\mu$M d-tubocurarine. These results indicate that human neuronal nicotinic AChR $\beta_2$ subunit transcripts generated from $\beta_2$ subunit DNA containing 5' untranslated sequence may be preferable to transcripts generated from $\beta_2$ DNA containing only a small portion of 5' untranslated sequence for expression of the $\alpha_3\beta_2$ receptors in oocytes.

EXAMPLE 4

Recombinant Expression of Human nNAChR Subunits in Mammalian Cells

I. Recombinant expression of human NAChR $\alpha_3$ and $\beta_4$ or $\alpha_7$ subunits in HEK 293 Cells:

Human embryonic kidney (HEK) 293 cells were transiently and stably transfected with DNA encoding human neuronal nicotinic AChR $\alpha_3$ and $\beta_4$ , or $\alpha_7$ subunits. Transient transfectants were analyzed for expression of nicotinic AChR using various assays, e.g., electrophysiological methods, $Ca^{2+}$-sensitive fluorescent indicator-based assays and [$^{125}$I]-$\alpha$-bungarotoxin-binding assays.

1. Transient Transfection of HEK Cells

Two transient transfection were performed. In one transfection, HEK cells were transiently co-transfected with DNA encoding $\alpha_3$ (plasmid pCMV-KE$\alpha$3) and $\beta_4$ (plasmid pCMV-KF$\beta$4) subunits. In the other transfection, HEK cells were transiently transfected with DNA encoding the $\alpha_7$ subunit (plasmid pCMV-KE$\alpha$7). In both transfections, ~$2\times10^6$ HEK cells were transiently transfected with 18 $\mu$g of the indicated plasmid(s) according to standard $CaPO_4$ transfection procedures [Wigler et al. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373-1376]. In addition, 2 $\mu$g of plasmid pCMV$\beta$gal (Clontech Laboratories, Palo Alto, Calif.), which contains the *Escherichia coli* $\beta$-galactosidase gene fused to the CMV promoter, were co-transfected as a reporter gene for monitoring the efficiency of transfection. The transfectants were analyzed for $\beta$-galactosidase expression by measurement of $\beta$-galactosidase activity [Miller (1972) Experiments in Molecular Genetics, pp.352-355, Cold Spring Harbor Press]. Transfectants can also be analyzed for $\beta$-

galactosidase expression by direct staining of the product of a reaction involving β-galactosidase and the X-gal substrate [Jones (1986) *EMBO* 5:3133-3142].

The efficiency of transfection of HEK cells with pCMV-KEα 3/pCMV-KEβ4 was typical of standard efficiencies, whereas the efficiency of transfection of HEK cells with pCMV-KEα7 was below standard levels.

2. Stable Transfection of HEK Cells

HEK cells were transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)]. Ten-cm plates, each containing one-to-two million HEK cells were transfected with 1 ml of DNA/calcium phosphate precipitate containing 9.5 μg pCMV-KEα3, 9.5 μg pCMV-KEβ4 and 1 μg pSV2neo (as a selectable marker). After 14 days of growth in media containing 1 μg/ml G418, colonies had formed and were individually isolated by using cloning cylinders. The isolates were subjected to limiting dilution and screened to identify those that expressed the highest level of nicotinic AChR, as described below.

3. Analysis of Transfectants

a. Fluorescent indicator-based assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including $Ca^{++}$, through the receptor channel. $Ca^{++}$ entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic $Ca^{++}$ levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular $Ca^{2+}$ concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying icotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK cells that were transiently or stably co-transfected with DNA encoding α3 and β4 subunits were analyzed for expression of functional recombinant nicotinic AChR using the automated fluorescent indicator-based assay. The assay procedure was as follows.

Untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) and HEK cells that had been co-transfected with pCMV-KEα3 and pCMV- KEβ4 were plated in the wells of a 96-well microtiter dish and loaded with fluo-3 by incubation for 2 hours at 20° C. in a medium containing 20 μM fluo-3, 0.2% Pluronic F-127 in HBS (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.62 mM $MgSO_4$, 6 mM glucose, 20 mM HEPES, pH 7.4). The cells were then washed with assay buffer (i.e., HBS). The antagonist d-tubocurarine was added to some of the wells at a final concentration of 10 μM. The microtiter dish was then placed into a fluorescence plate reader and the basal fluorescence of each well was measured and recorded before addition of 200 μM nicotine to the wells. The fluorescence of the wells was monitored repeatedly during a period of approximately 60 seconds following addition of nicotine.

The fluorescence of the untransfected HEK cells (or HEK cells transfected with pCMV-T7-2) did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express functional recombinant AChR that are activated by nicotine and blocked by d-tubocurarine.

b. α-Bungarotoxin binding assays

HEK293 cells transiently transfected with pCMV-KEα7 were analyzed for [$^{125}$I]-α-bungarotoxin (BgTx) binding. Both whole transfected cells and membranes prepared from transfected cells were examined in these assays. Rat brain membranes were included in the assays as a positive control.

Rat brain membranes were prepared according to the method of Hampson et al. (1987) *J. Neurochem* 49:1209. Membranes were prepared from the HEK cells transfected with pCMV-KEα7 and HEK cells transiently transfected with plasmid pUC19 only (negative control) according to the method of Perez-Reyes et al. (1989) *Nature* 340:233. Whole transfected and negative control cells were obtained by spraying the tissue culture plates with phosphate-buffered saline containing 0.1% (w/v) BSA. The cells were then centrifuged at low speed, washed once, resuspended in assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, 0.1% (w/v)BSA, 0.05% (w/v) bacitracin and 0.5 mM PMSF, pH 7.5) and counted.

Specific binding of [$^{125}$I]-α-BgTx to rat brain membranes was determined essentially as described by Marks et al. (1982) *Molec. Pharmacol.* 22:554-564, with several modifications. The membranes were washed twice in assay buffer. The assay was carried out in 12×75 mm polypropylene test tubes in a total volume of 0.5 ml assay buffer. The membranes were incubated with 10 nM [$^{125}$I]-α-BgTx (New England Nuclear, Boston, Mass.) for one hour at 37° C. The assay mixtures were then centrifuged at 2300×g for 10 minutes at 4× C. The supernatant was decanted and the pellets were washed twice with 2 ml aliquots of ice-cold assay buffer. The supernatants were decanted again and the radioactivity of the pellets was measured in a γ-counter. Non-specific binding was determined in the presence of 1 μM unlabeled α-BgTx. Specific binding was determined by subtracting nonspecific binding from total binding. Specific

binding of [$^{125}$I]-α-BgTx to membranes prepared from transfected and negative control cells was determined as described for determining specific binding to rat brain membranes except that the assay buffer did not contain BSA, bacitracin and PMSF. Specific binding of [$^{125}$I]-α-BgTx to transfected and negative control whole cells was determined basically as described for determining specific binding to rat brain membranes.

[$^{125}$I]-α-BgTx binding was evaluated as a function of membrane concentration and as a function of incubation time. [$^{125}$I]-α-BgTx binding to rat brain membranes increased in a linear fashion with increasing amounts of membrane (ranging between 25-500 μg). The overall signal-to-noise ratio of binding (i.e., ratio of total binding to non-specific binding) was 3:1. Although some binding of [$^{125}$I]-α-BgTx to transfected cell membranes was detected, it was mostly non-specific binding and did not increase with increasing amounts of membrane. [$^{125}$I]-α-BgTx binding to the transfectants and negative control cells appeared to be similar.

To monitor [$^{125}$I]-α-BgTx binding to rat brain membranes and whole transfected and negative control cells, 300 μg of membrane or 500,000 cells were incubated with 1 nM or 10 nM [$^{125}$I]-α-BgTx, respectively, at 37° C. for various times ranging from 0-350 min. Aliquots of assay mixture were transferred to 1.5 ml microfuge tubes at various times and centrifuged. The pellets were washed twice with assay buffer. [$^{125}$I]-α-BgTx binding to rat brain membranes increased with time and was maximal after three hours. The binding profiles of the transfected and negative control cells were the same and differed from that of rat brain membranes.

Recombinant Expression of Human nAChR Subunits (multimeric subunit combinations) in Mammalian Cells

II. (a) Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic nAChR containing multimeric Subunits.

Isolated cDNAs encoding human neuronal nAChRs were incorporated into vectors for use in expressing the subunits in mammalian host cells.

A. Construct for expression of a human nAChR α3 subunit.

Construct pCMV-KEα3 (Fig. 12) is described in U.S. Patent 5,837,489, the contents of which are incorporated by reference herein in ther entirety. was digested with HindIII and NotI to release a 1.7 kb DNA fragment containing the entire α3 coding region. The expression construct pcDNA3-KEα3 was prepared by ligating the 1.7 kb α3 DNA fragment from pCMV-KEα3 into vector HindIII and NotI digested pcDNA3 (Invitrogen).

B. Construct for expression of a human nAChR α5 subunit.

DNA fragments encoding portions of a human nAChR α5 subunit were ligated together to generate a full-length α5 subunit coding sequence contained in plasmid pcDNA1/Amp-KEα5.5F. This construct was modified by replacing the 5'untranslated sequence of the α5 subunit DNA with a consensus ribosome binding site, RBS, (5'-GCCACC-3', see Kozak (1987)

Nucl. Acids Res. 15:8225-8148) to generate pcDNA1/Amp-KEα5RBS). Construct pcDNA1/Amp-KEα5RBS was digested with BamHI and EcoRI to release a 1.7 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of α5 and also containing the entire α5 coding region. Construct pcDNA3-KEα5RBS was prepared by digestion of pcDNA3 with BamHI and EcoRI followed by ligation of the 1.7 kb α5 DNA fragment. The pcDNA3-KEα5RBS construct was then digested with Asp718I and BstX1 to release a 1.7kb fragment containing the entire α5 coding sequence with the RBS immediately 5' to the α5 sequence. This fragment was ligated into expression vector pHOOK3 (Invitrogen) which had been digested with Asp718I and BstXI to generate the expression construct pHOOK3-KEα5RBS (Fig. 13).

C. Construct for expression of a human nAChR β2 subunit.

Construct pCMV-KEβ2 (described in Patent 5,910,582) was modified by replacing the 5'untranslated sequence of the β2 subunit DNA with a consensus ribosome binding site (5'-GCCACC-3', see Kozak (1987) Nucl. Acids Res. 15:8225-8148) to generate pCMV-KEβ2RBS. The expression vector pCMV-KEβ2RBS was digested with BglII and EcoRI to release a 2.2 kb DNA fragment containing the consensus ribosome binding site immediately 5' to the translation initiation codon of β2 and also containing the entire β2 coding region. This 2.2 kb DNA fragment was ligated into expression vector pcDNA3 that had been digested with BamHI and Eco RI. The BamHI site is compatible with BglII and this ligation generated expression construct pcDNA3-KEβ2RBS (Fig. 14).

II. (b) Recombinant Expression of the Human α3β2α5 nAChR in HEK293 cells.

Human embryonic kidney cells (HEK 293) were stably co-transfected with DNA encoding human neuronal nAChR α3, β2 and α5 and analyzed for expression of nAChRs using various assays, for example, calcium sensitive fluorescent indicator-based assays and electrophysiological methods.

1. Stable co-transfection of HEK293 cells with human α3, β2 and α5 nAChRs.

a. Expression Strategy.

The α5 nAChR is non-functional when expressed with either another α subunit or another β subunit. In order to develop a functional 3-way nAChR that includes the α5 subunit, α5 was co-expressed with both α3 and β2. The antibiotic selection strategy was designed to take advantage of the lack of function of co-expression of either α3α5 or α5β2. Even though these combinations would survive the antibiotic selection, they would be non-functional. Using this expression strategy, the only possible nAChR subunit combination surviving antibiotic selection and having functional responses would be α3β2α5. The expression strategy for the generation of this subunit combination is described in detail below.

The α3 was cloned into pcDNA3 (Invitrogen) that encodes a neomycin resistance gene permitting tolerance to the antibiotic G418. The β2 subunit was also cloned into pcDNA3. The

α5 subunit was cloned into the expression vector pHOOK3 (Invitrogen) which encodes the Zeocin™ (Invitrogen) resistance gene that allows tolerance to the antibiotic Zeocin™. By this strategy, cells stably expressing the α5 nAChR and α3 or α5 and β2 could survive in a selection culture medium containing both G418 and Zeocin™. However, stable expression of α3, α5 and β2 would be required for function.

b. Recombinant Expression of Human α3β2α5 nAChRs.

HEK293 cells were stably co-transfected with DNA encoding human neuronal nAChRs α3, β2 and α5 using the lipofection transfection procedure (Current Protocols in Molecular Biology, Volume 1, 9.4.1 – 9.4.5 and 9.5.1 – 9.5.6, the contents of which are incorporated by reference herein). HEK293 cells were harvested and plated onto 10 cm tissue culture plates that were coated with poly-D-lysine. The HEK293 cells were plated at a concentration of 1.2 million cells per plate, 24 hours prior to transfection. Two micrograms of DNA encoding α3 (mammalian expression vector pcDNA3-KEα3), 2 μg of DNA encoding β2 (pcDNA3-KEβ2RBS) and 2 μg of DNA encoding α5 (pHOOK3-KEα5RBS) were diluted in 300 μl of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20μl of LipofectAMINE™ Reagent (Gibco-BRL) for 15 minutes. The HEK293 cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the HEK293 cells. The overlaid cells were incubated for 5 hours in an incubator at 37°C, in a humidified atmosphere containing 5% carbon dioxide. Cell plates were washed twice with 5mls of complete media (DMEM, 6% iron-supplemented calf serum, 2 mM glutamine, 100 units per ml of penicillin and 100 μg/ ml streptomycin) then overlaid with 10ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 100 μg/ml of G418 plus 40μg/ml Zeocin™ was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 20 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations (Reference to analysis of transfectants, section 2). Two parental cell lines, 83-13 and 83-19 exhibited robust expression of the 3-way combination in functional calcium assays and both were subcloned by limiting dilution.

Thirty seven subclones from parental cell line 83-19 were screened in the fluorescence-based calcium assay. Sixteen subclones were positive in this assay and showed epibatidine-induced increases in internal calcium. Twelve subclones from parental cell line 83-13 were screened in the fluorescence-based calcium assay and five subclones were positive. Four subclones, including subclone 83-19-15 were selected based on activity in calcium assays.

83-19-15 was further subcloned by limiting dilution, and 18 subclones were screened for acetylcholine-induced increases in internal calcium. Four subclones (83-19-15-26, 83-19-15-27, 83-19-15-42 and 83-19-15-48 were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for acetylcholine-induced increases in internal calcium at two-weekly intervals for approximately 15 weeks.

Subclone 83-19-15-27 was selected based on the stable functional response to low doses of acetylcholine (1 μM) observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A3B2A5 after validation in this assay (example 5, protocol A) .

2. Analysis of Transfectants

a. Fluorescent indicator-based assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including $Ca^{++}$, through the receptor channel. $Ca^{++}$ entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic $Ca^{++}$ levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular $Ca^{2+}$ concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying icotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

HEK293 cells that were stably transfected with DNA encoding the human α3β2α5 subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Briefly, untransfected HEK293 cells and HEK293 cells that had been transfected with DNA encoding human α3, α5 and β2 nAChRs were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a cell density of 75,000 to 200,000 cells per well. Cells were grown in an incubator at 37°C for 2-3 hours, then transferred to an incubator maintained at 28°C. Forty-eight hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 21.8 mM $CaCl_2$ , 1μM atropine, 6 mM glucose and 20 mM HEPES-NaOH pH7.4. Washed cells were incubated with 20 μM fluo-3-acetoxymethylester containing 0.16% pluronic F-127 at 22°C for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250 μl HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.).

Ten basal fluorescence readings were recorded prior to addition of agonist (either 100 nM epibatidine, or 1 μM acetylcholine). Responses after the addition of epibatidine were recorded for approximately 60 sec. Maximal fluorescence ($F_{max}$) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ($F_{min}$) was determined after subsequent quenching with 10 mM $MnCl_2$. Calculation of $[Ca^{2+}]_i$ was performed as described by Kao *et al.* (1989). Cellular responses were quantitated by calculating either the ratio of peak $[Ca^{2+}]_i$ after agonist addition to the basal $[Ca^{2+}]_i$ prior to agonist addition, or by the difference between peak $[Ca^{2+}]_i$ and basal $[Ca^{2+}]_i$.

The fluorescence of the untransfected HEK cells did not change after addition of nicotine. In contrast, the fluorescence of the co-transfected cells, in the absence of d-tubocurarine, increased dramatically after addition of nicotine to the wells. This nicotine-stimulated increase in fluorescence was not observed in co-transfected cells that had been exposed to the antagonist d-tubocurarine. These results demonstrate that the co-transfected cells express the above referenced functional recombinant multimeric AChR subunit combination that were activated by nicotine and blocked by d-tubocurarine.

b. Characeristics of the stable cell line A3B2A5 that expresses the human α3β2α5 nAChR.

Pharmacological analysis of agonist-induced increases in internal calcium using the fura-2 calcium assay (Protocol A, *infra*, Reference to SpeedReader patent?) showed the expression of two populations of nAChRs in A3B2A5 cells: one population displayed high sensitivity to some nAChR agonists while the second showed a sensitivity to agonists indistinguishable from that observed in cell line A3B2 (which expresses human α3β2 nAChRs). The high affinity site in A3B2A5 cells displays a 200- to 6000-fold lower $EC_{50}$ value for the agonists acetylcholine (ACh), nicotine and cytisine compared to α3β2 nAChRs. Figure 9a and 9b illustrate some of the pharmacology of the A3B2A5 cell line. The changes in agonist sensitivity result in a rank order of agonist potency for A3B2A5 that differs from that of A3B2 and thus demonstrates the presence of a novel receptor (α3β2α5) in cell line A3B2A5. In whole-cell voltage-clamped A3B2A5 cells, we found that the desensitization kinetics of currents elicited by low doses of ACh are significantly slower in A3B2A5 cells than A3B2 cells (Protocol B) (Fig. 10). The differences in biophysical properties of A3B2A5 and A3B2 also indicate the expression of a novel receptor, the α3β2α5 nAChR, in cell line A3B2A5 and these are illustrated in Figure 9b. The homogeneity of the cell line was verified by single-cell imaging of agonist-induced increases in intracellular free calcium concentration (Protocol C). Co-precipitation experiments demonstrated the co-assembly of the α5 nAChR with α3 and with β2 (protocol D, figure 11).

The protocols for the above referenced data is presented hereafter.

A. Fluorescence-based calcium assays using Fura-2.

A cell line, A3B2A5, stably transfected with DNAs encoding human α3, α5, and β2 receptors is plated in black-walled 96-well plates, grown 2 to 3 hours at 37°C and then 2 days at 28 °C. At the start of the assay, assay the plates are washed with in HEPES buffered saline (HBS) containing 1 μM atropine (HBSA) (wash cycle = aspirate, dispense x 3) to leave 180 μl residual HBSA per well. Then a background measurement of a sample plate is taken by the SpeedReader

for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. Twenty µl of 10 µM fura-2 dye containing is then added to each well and incubated with the cells at ambient temperature for one hour to two hours. After dye loading the free dye is washed from the wells with HBSA to leave 180 µl residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM $CaCl_2$ and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the Ca-indicating dye fura-2. After the first 20 frames are collected 20 µl of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute Ca concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for Ca. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

B. Electrophysiological Analysis

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

HEK293 cells stably transfected with DNA encoding the human α3, β2 and α5 subunits were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents. HEK293 cells stably expressing human α3, β2 and α5 nAChRs were plated at a density of 1.5 x $10^5$ cells/35-mm dish on poly-D-lysine-coated glass coverslips (0.1 mg/ml, SIGMA) and incubated at 37°C for 2-3 hours, then for 48 hours at 28°C. Recordings were performed with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at -100 mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of ≈3.0 ml/min (110 µl recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Agonist, dissolved in Ringer's solution, was applied for 200-500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics).

The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant $\tau$=0.7 ms, with steady state reached <3 ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

B. Single cell calcium imaging assays using Fura-2

Cells stably transfected with DNAs encoding human $\alpha$3, $\beta$2 and $\alpha$5 nAChR subunits were plated on poly-D-lysine-coated glass coverslips at a density of 3 x $10^5$ cells/35 mm dish and grown at 28°C. Forty-eight hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1μM fura-2-AM (Molecular Probes, Inc.) for 0.5 - 1 h and washed with mammalian Ringer's solution (see example 4, 2c for composition) to remove excess dye. Cells were transferred to a recording chamber (110 μl, Warner Instruments), and continuously superfused with HBK containing 21.8 mM $CaCl_2$ and1μM atropine at a rate of 8 - 10 ml/min. Agonist was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

C. Western analysis and immunoprecipitation to demonstrate co-expression of $\alpha$3, $\beta$2 and $\alpha$5 nAChR subunit proteins.

Cells stably transfected with DNA encoding human $\alpha$3, $\beta$2 and $\alpha$5 nAChR subunits were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete™, Boehringer Mannheim, Indianapolis, IN) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000 x g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoprecipitation experiments, 200 μg of membranes were immunoprecipitated with 20 μg of a sheep anti-rat $\alpha$3 polyclonal antibody (Bethyl Laboratories, or 2 μg a rabbit anti-human $\beta$2 polyclonal antibody (MRL San Diego) overnight at 4°C. The antibody-antigen complexes were affinity-purified using Protein G sepharose, incubated overnight at 4°C then solubilized in SDS sample buffer. For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex) containing 5% 2-mercaptoethanol and heated at 65°C for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, IL). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).
The human $\alpha$5 protein was detected with a sheep anti-rat $\alpha$5 antibody (Bethyl Laboratories). The $\alpha$5 antibody was diluted to 15 μg/ml in blocking buffer and incubated with the nitrocellulose

membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-sheep IgG (Cappell Antibodies) diluted 1:1000 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

The above strategy may be employed in expressing any one of the following multimeric subunit combinations of the alpha and beta subunit sof nAChR, especially when the nucleic acid molecule encoding each individual nAChR subunit is disclosed herein. In view of the above data, it is not seen why the proposed combinations appearing below would not act in a manner similar to the multimeric subunit combination discussed immediately above.

α2β4α6
α3β4α6
α4β4α5
α4β4α6
α4β2α5
α4β2β3
α3β2α6β3
α2β4α5
α2β2α5
αXβ2β4, where X refers to one or more of the α subunts disclosed herein.
αXβ2β3β4, where X refers to one or more of the α subunts disclosed herein
aXb2b3. where X refers to one or more of the α subunits disclosed herein
α2β2α6
α3β2α6
α4β2α6

Five-way combinations of subunits, represented by the general formula $\alpha_n\beta_m$, wherein n and m are each 0-5 (where the α subunit is one or more of $\alpha_1$ thru $\alpha_7$ and β is any one or more of $\beta_2$, $\beta_3$ or $\beta_4$ are also contemplated by the present invetion. Likewise, four-way combinations are also a feature of the invention.

III. Recombinant Expression of the Human α7 nAChR in a non-human cell line

A. Construct for Expression of recombinant Human nNAChR $\alpha_7$ in a Non-human Host Cell Line

The isolated cDNA encoding human neuronal α7 AChR was incorporated into the expression vector pcDNA3 (Invitrogen) for use in expressing the α7 subunit in the $GH_4C_1$ host cell line. The expression vector, pcDNA3-KEα7RBS was constructed as described below.

Construct pCMV-KEα7 was digested with BamHI and XhoI to release a 1.8kb DNA fragment containing a consensus ribosome binding site (RBS) immediately 5' to the translation initiation codon of α7 and also containing the entire α7 coding region. pGEM/KEα7RBS was prepared by

ligating this 1.8kb DNA fragment into BamHI, XhoI digested pGEM-7Zf(+), (Promega). pGEM/KEα7RBS was digested with BamHI and XhoI to release the 1.8kb DNA fragment containing the RBS and α7 coding region. pcDNA3-KEα7RBS was prepared by ligating the 1.8kb fragment from pGEM/KEα7RBS into BamHI and XhoI digested pcDNA3.

B. Recombinant Expression of the Human α7 nAChR in $GH_4C_1$ cells.

$GH_4C_1$ cells, derived from rat pituitary tumor tissue, were stably transfected with DNA encoding human neuronal nAChR α7 and analyzed for expression of nAChRs using various assays, for example calcium sensitive fluorescent indicator-based assays, [$^{125}$I] bungarotoxin binding and electrophysiological methods.

1. Stable Transfection of $GH_4C_1$ cells with the human α7 nAChR.

$GH_4C_1$ cells were stably transfected with DNA encoding human neuronal nAChR α7 using the lipofection transfection procedure (Current Protocols in Molecular Biology, Volume 1, 9.4.1 – 9.4.5 and 9.5.1 – 9.5.6, incorporated herein by reference).

$GH4C_1$ cells were harvested using Cell Dissociation Buffer (Sigma) and plated onto 10 cm tissue culture plates coated with poly-d-lysine at a concentration of 1.2 million cells per plate, 24 hours prior to transfection. Six micrograms of the α7 expression vector, pcDNA3-KEα7RBS were diluted in 300 µl of Dulbecco's Modified Eagle Medium (DMEM) and combined with 20µl of LipofectAMINE™ Reagent (Gibco-BRL) for 15 minutes. The $GH_4C_1$ cells were washed twice with DMEM. This DNA/LipofectAMINE mixture was further diluted into 5.3 ml of DMEM and overlaid onto the $GH_4C_1$ cells. The overlaid cells were incubated for 5 hours in an incubator at 37°C, in a humidified atmosphere containing 6% carbon dioxide. Cell plates were washed twice with 5mls of Ham's F-10 nutrient mixture (GibcoBRL) containing 10% fetal bovine serum, 100 units per ml of penicillin and 100 µg/ ml streptomycin then overlaid with 10ml of complete medium and placed in an incubator for 48 hours.

Forty-eight hours post-transfection, cell plates were split at a 1:4 ratio, generating four culture plates. Twenty hours later, complete medium containing 500 µg/ml of G418 was added to the cells for 14 days. Medium was replaced every 2 to 4 days. After this period, colonies had formed on the plates and were isolated using trypsin-soaked circles of sterile filter paper. 24 isolates were cultured, 18 survived and were expanded for functional assay using fluorescence-based measurements of internal calcium concentrations as descried in Example 4 above.

Clones were also screened in a radioligand binding assay using [$^{125}$I]- bungarotoxin. See example 4. Electrophysiological recordings (similar to the procedue outlined in Example 4) also demonstrated currents with biophysical properties characteristic of the α7 receptor. Parental cell line G1-9 exhited robust expression in both functional calcium and electrophysiological assays and in binding assays. The G1-9 parental cell line was subcloned by limiting dilution.

Twenty eight subclones from G1-9 were screened in the fluorescence-based calcium assay. Ten subclones were positive in this assay and showed epibatidine-induced increases in internal

calcium. An additional binding assay, similar to that outlined above, identified thirteen positive subclones.

Five subclones, including subclone G1-19-15 were selected based on activity in both calcium and binding assays.

G1-9-15 was further subcloned by limiting dilution, subclones were screened for epibatidine-induced increases in internal calcium. Four subclones, G1-9-15-8, G1-9-15-18, G1-9-15-28 and G1-9-15-35 were selected based on a positive functional response in this assay. These subclones then entered a stability study where they were monitored for functional response in the calcium assay at two-weekly intervals for approximately 15 weeks.

Subclone G1-9-15-8 was selected based on the stable functional response observed during the stability study. This cell line was confirmed to have acceptable responses as a random screening target in the high throughput screening assay and renamed A7 after validation in this assay .

2. Analysis of Transfectants

a. Fluorescence-based measurements of internal calcium concentrations.

$GH_4C_1$ cells that were stably transfected with DNA encoding the human α7 subunit were analyzed for expression of functional recombinant nAChRs using the automated fluorescent indicator-based assay.

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including $Ca^{++}$, through the receptor channel. $Ca^{++}$ entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic $Ca^{++}$ levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, Oreg.), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular $Ca^{2+}$ concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying icotinic AChR has been described in commonly assigned pending U.S. Pat. application Ser. No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

Untransfected $GH_4C_1$ cells and $GH_4C_1$ cells that had been transfected with pcDNA3-KEα7RBS were plated in the wells of a poly-D-lysine coated 96-well microtiter dish at a cell density of

75,000 to 200,000 cells per well. Twenty four hours after plating, cell culture medium was decanted and cells washed with an assay buffer (HBK) containing 155 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$ , 1μM atropine, 6 mM glucose and 20 mM Hepes-NaOH pH7.4. Washed cells were incubated with 20 μM fluo-3-acetoxymethylester containing 0.16% pluronic F-127 at 22°C for 2 hours in the dark. Dye not taken up by cells was removed by aspiration followed by washing with 250 μl HBK. Fluorescence measurements were performed at 0.33 sec intervals using a 96-well microtiter plate-reading fluorometer (Cambridge Technology, Inc.). Cells were incubated for 10 minutes with 3 μM FPL 64176 and ten basal fluorescence readings were recorded prior to addition of 1 μM epibatidine. Responses after the addition of epibatidine were recorded for approximately 60 sec. Alpha-bungarotoxin was tested after a preincubation period of 5 - 10 min. Maximal fluorescence ($F_{max}$) was determined after lysing the cells with 0.25% Triton X-100, and minimal fluorescence ($F_{min}$) was determined after subsequent quenching with 10 mM $MnCl_2$. Calculation of $[Ca^{2+}]_i$ was performed as described by Kao *et al.* (1989). Cellular responses were quantitated by calculating either the ratio of peak $[Ca^{2+}]_i$ after agonist addition to the basal $[Ca^{2+}]_i$ prior to agonist addition, or by the difference between peak $[Ca^{2+}]_i$ and basal $[Ca^{2+}]_i$.

b. α-Bungarotoxin Binding Assays

Untransfected $GH_4C_1$ cells and $GH_4C_1$ cells that were stably transfected with DNA encoding the human α7 subunit were analyzed for [$^{125}$I]-α-bungarotoxin binding. The assay procedure was as follows.

Cells were incubated with 1nM [$^{125}$I]- α BTX in culture media for 2 hours at room temperature. Non-specific binding was determined in the presence of 1 μM unlabeled toxin. The assays were terminated by aspiration of the culture media and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4x1ml washes of ice cold binding assay buffer (50 mM tris, 140 mM NaCl, 5 nM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4). Filter disks were transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail and radioactivity counted using a Beckman 6500 scintillation spectrometer.

c. Electrophysiological Analysis of $GH_4C_1$ cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA (human α7 subunit)

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques.

$GH_4C_1$ cells stably transfected with DNA encoding the human $\alpha_7$ subunit were analyzed electrophysiologically for the presence of nAChR agonist-dependent currents. GH4C1 cells stably expressing human α7 nAChRs were plated at a density of 1.5 x $10^5$ cells/35-mm dish on

collagen-coated glass coverslips (rat collagen I, Becton Dickinson) treated with an additional coating of poly-D-lysine (0.1 mg/ml, SIGMA). Recordings were performed with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell voltage-clamp configuration. Membrane potential was held at -100 mV. The standard external recording solution (mammalian Ringer's) consisted of (in mM) 160 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 0.001 atropine, and 5 HEPES, pH 7.3. Ringer's solution was superfused at a rate of ≈3.0 ml/min (110 $\mu$l recording chamber). The recording pipette solution was composed of 135 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.3 (with or without 4 mM Mg-ATP). Experiments were performed at room temperature. Nicotine (100-300 $\mu$M), dissolved in Ringer's solution, was applied for 200-500 ms using a fast application system, consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics). The speed of solution exchange between control and nicotine-containing solutions, measured as the open-tip response, displays a time constant $\tau$=0.7 ms, with steady state reached <3 ms. Data were digitized at 6.7 kHz and filtered at 2 kHz on line. Data analysis was performed using pClamp software (Axon Instruments).

EXAMPLE 5

Characterization of Cell Lines Expressing nNAChRs

Recombinant cell lines generated by transfection with DNA encoding human neuronal nicotinic AChRs, such as those described in Example 3 can be further characterized using one or more of the following methods.

A. Northern or slot blot analysis for expression of α- and/or β-subunit encoding messages

Total RNA is isolated from ~$1\times10^7$ cells and 10-15 $\mu$g of RNA from each cell type is used for northern or slot blot hybridization analysis. The inserts from human neuronal NAChR-encoding plasmids can be nick-translated and used as probe. In addition, the β-actin gene sequence (Cleveland et al. (1980) Cell 20:95-105) can be nick-translated and used as a control probe on duplicate filters to confirm the presence or absence of RNA on each blot and to provide a rough standard for use in quantitating differences in α- or β-specific mRNA levels between cell lines. Typical northern and slot blot hybridization and wash conditions are as follows:

hybridization in 5× SSPE, 5× Denhardt's solution, 50% formamide, at 42° C. followed by washing in 0.2× SSPE, 0.1% SDS, at 65° C.

B. Nicotine-binding assay

Cell lines generated by transfection with human neuronal nicotinic AChR α- or α- and β-subunit-encoding DNA can be analyzed for their ability to bind nicotine, for example, as compared to control cell lines: neuronally-derived cell lines PC12 (Boulter et al., (1986), supra; ATCC #CRL1721) and IMR32 (Clementi, et al. (1986); Int. J. Neurochem. 47:291-297; ATCC #CCL127), and muscle-derived cell line BC3H1 (Patrick, et al, (1977); J. Biol. Chem. 252:2143-2153. Negative control cells (i.e., host cells from which the transfectants were prepared) are also included in the assay. The assay is conducted as follows:

Just prior to being assayed, transfected cells are removed from plates by scraping. Positive control cells used are PC12, BC3H1, and IMR32 (which had been starved for fresh media for seven days). Control cell lines are removed by rinsing in 37° C. assay buffer (50 mM Tris/HCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 120 mM NaCl, 3 mM EDTA, 2 mg/ml BSA and 0.1% aprotinin at pH7.4). The cells are washed and resuspended to a concentration of $1\times10^6$/250 $\mu$l. To each plastic assay tube is added 250 $\mu$l of the cell solution, 15 nM $^3$H-nicotine, with or without 1 mM unlabeled nicotine, and assay buffer to make a final volume of 500 $\mu$l. The assays for the transfected cell lines are incubated for 30 min at room temperature; the assays of the positive control cells are incubated for 2 min at 1° C. After the appropriate incubation time, 450 $\mu$l aliquots of assay volume are filtered through Whatman GF/C glass fiber filters which has been pretreated by incubation in 0.05% polyethyleneimine for 24 hours at 4° C. The filters are then washed twice, with 4 ml each wash, with ice cold assay buffer. After washing, the filters are dried, added to vials containing 5 ml scintillation fluid and radioactivity is measured.

C. $^{86}$Rb ion-flux assay

The ability of nicotine or nicotine agonists and antagonists to mediate the influx of $^{86}$Rb into transfected and control cells has been found to provide an indication of the presence of functional AChRs on the cell surface. The $^{86}$Rb ion-flux assay is conducted as follows:

1. The night before the experiment, cells are plated at $2\times10^6$ per well (i.e., 2 ml per well) in a 6-well polylysine-coated plate.

2. The culture medium is decanted and the plate washed with 2 ml of assay buffer (50 mM HEPES, 260 mM sucrose, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgsO_4$, 5.5. mM glucose) at room temperature.

3. The assay buffer is decanted and 1 ml of assay buffer, containing 3 $\mu$Ci/ml $^{86}$Rb, with 5 mM ouabain and agonist or antagonist in a concentration to effect a maximum response, is added.

4. The plate is incubated on ice at 1° C. for 4 min.

5. The buffer is decanted into a waste container and each well was washed with 3 ml of assay buffer, followed by two washes of 2 ml each.

6. The cells are lysed with 2x0.5 ml of 0.2% SDS per well and transferred to a scintillation vial containing 5 ml of scintillation fluid.

7. The radioactivity contained in each vial is measured and the data calculated.

Positive control cells provided the following data in this assay:

| | PC12 | IMR32 |
|---|---|---|

| | $EC_{50}$ | Maximum response | $EC_{50}$ | Maximum response |
|---|---|---|---|---|
| Agonist | | | | |
| nicotine | 52 $\mu$M | 2.1 X[a] | 18 $\mu$M | 7.7 X[a] |
| CCh* | 35 $\mu$M | 3.3 X[b] | 230 $\mu$M | 7.6 X[c] |
| cytisine | 57 $\mu$M | 3.6 X[d] | 14 $\mu$M | 10 X[e] |
| Antagonist | | | | |
| d-tubocurarine | 0.81 $\mu$M | | 2.5 $\mu$M | |
| mecamylamine | 0.42 $\mu$M | | 0.11 $\mu$M | |
| hexamethonium | nd[f] | | 22$\mu$M | |
| atropine | 12.5 $\mu$M | | 43 $\mu$M | |

*CCh = carbamylcholine
[a]200 $\mu$M nicotine
[b]300 $\mu$M CCh
[c]3 mM CCh
[d]1 mM cytisine
[e]100 $\mu$M cytisine
[f]nd = not determined

D. Electrophysiological Analysis of Mammalian Cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques. In preferred embodiments, transfected mammalian cells or injected oocytes are analyzed electrophysiologically for the presence of AChR agonist-dependent currents.

Example 6

Characterization of $GH_4C_1$ Cells Stably Expressing the Human $\alpha$7 nAChR

The cell line A7 that stably expressed the human $\alpha$7 nAChR was characterized in multiple assays that are described below.

Dose response curves to reference nicotinic agonists nicotine and acetylcholine were obtained for cell line A7 using the fura-2 based calcium assay. See protocol A *infra.*

Refering to Figure 3, the $EC_{50}$ for nicotine was 2 μM and for acetylcholine was 7 μM. This is in agreement with that reported for the α7 nAChR ( Peng et al (1993) Mol Pharmacol. 45:546-554).

Data on electrophysiological characterization using whole-cell voltage-clamped A7 cells is depicted in Figure 4, which show rapidly desensitizing currents that are consistent with those reported for α7 nAChRs. The protoclos for these experimenst were the same as those described in Examples 3 and 4 above. In these studies 90% to 100% of voltage-clamped A7 cells responded to the application of 300 μM nicotine.

Single cell calcium imaging of the A7 cell line (Figure 6) (protocol B, *infra*) supports the conclusion that individual cells in this cell line (A7) respond to 10 μM epibatidine in a homogenous manner.

In radioligand binding studies (protocol C, *infra*) methyllycaconitine (MLA) displaced [$^3$H]-MLA binding from the α7 nAChRs in cell line A7 with an $IC_{50}$ of 4 nM, similar to the $IC_{50}$ value obtained with α-bungarotoxin (3 nM). These $IC_{50}$ values are similar to published affinities (for example, Davies *et al.* 1999, Neuropharmacology 38:679). α-bungarotoxin displaced approximately 65% of the [$^3$H]-MLA binding in A7. Cells are permeable to MLA but not to α-bungarotoxin under these assay conditions. This therefore demonstrates that 65% of the α7 nAChRs in cell line A7 are expressed on the plasma membrane (i.e. at the cell surface). This data is illustrated in Figure 5.

A molecular characterization was undertaken to demonstrate the expression of α7 nAChR protein and α7 mRNA in the stable cell line A7. Western analysis using an α7-specific antibody demonstrated that cell line A7 expressed protein of approximately 54 kDa. Protein prepared from the untransfected $GH_4C_1$ cell line does not show any hybridization with this antibody. Refer to Figure 7.

Northern analysis of total RNA prepared from A7 cells showed that these cells express an RNA species that hybridizes with a subunit specific DNA probe. The hybridizing band has a molecular weight of approximately 2.4 kb. No hybridizing species was detected in untransfected $GH_4C_1$ cells. Refer to Figure 8.

The characterizations of stable cell line A7 described above were generated using the following protocols .

A. Fluorescence based calcium assays using Fura-2
A cell line A7 stably transfected with the human α7 nAChR receptor is plated in black-walled 96-well plates and grown at 37°C. Twenty-four hours later, the plates are washed with in HEPES buffered saline (HBS) containing 1 μM atropine (HBSA) (wash cycle = aspirate, dispense x 3) to

leave 180 µl residual HBSA per well. At the start of the assay, a background measurement of a sample plate was taken by the SpeedReader for 20 frames alternating the excitation light between 350 and 385 nm at four hertz. See U.S. Patent number 5,670,113 and 6,057,114, each of which is incorportaed by reference herein in their entirety. Twenty µl of 10 µM fura-2 dye containing 3 µM FPL-64176 is then added to each well and incubated with the cells at ambient temperature for one to two hours. After dye loading the free dye is washed from the wells with HBSA containing 0.5 µM FPL-64176 to leave 180 µl residual buffer per well. Two minutes after washing, a kinetic reading is taken while the test chemicals are added. The test compounds are prepared in HBSA containing 80 mM $CaCl_2$ and 1% DMSO. The kinetic reading is composed of 140 frames, alternating between 350 and 385 as in the 20 frame background reading. However, the first 20 frames of the kinetic reading are taken before test chemical addition. The difference between these 20 frames and the background give the fluorescence due to the calcium-indicating dye fura-2. After the first 20 frames are collected 20 µl of the test compound is dispensed from a 96-channel pipettor to the entire plate at once without halting the reading. The remainder of the 120 frames of data measure the response.

Absolute calcium concentrations are not calculated from these readings, rather the directly measured fluorescence ratio is used as a surrogate for calcium. The fluorescence ratio is calculated as dye fluorescence generated by excitation at 350 nm divided by dye fluorescence generated by excitation at 385 nm. The raw activity in a well is calculated as the maximum fluorescence ratio after compound addition divided by the average fluorescence ratio before compound addition.

B. Single cell calcium imaging assays using Fura-2

Cells stably transfected with the human α7 nAChR were plated on poly-D-lysine-coated glass coverslips at a density of 3 x $10^5$ cells/35 mm dish. Twenty four hours later, imaging experiments were performed at room temperature, using a Nikon TE200 inverted microscope attached to a DeltaRAM imaging System (Photon Technology International). Cells were incubated with 1µM fura-2-AM (Molecular Probes, Inc.) for 0.5 - 1 h and washed with mammalian Ringer's solution (see example above re: the ephys composition of this buffer eg Ringers (in mM) 160 NaCl, 5 KCl, 1 MgCl etc.) to remove excess dye. Cells were transferred to a recording chamber (110 µl, Warner Instruments), and continuously superfused with HBK containing 1µM atropine at a rate of 8 - 10 ml/min. 10µM epibatidine was applied by switching between reservoirs. Cells were alternatively excited at 360 and 381 nm (0.5 Hz) to determine ratio images.

C. Radioligand Binding Studies

GH4C1 cells stably expressing α7 were plated in 96-well microtiter plates at a density of 200,000 cells per well. Twenty-four hours later, cells were washed in assay buffer (50 mM Tris, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), and incubated with 1 nM [$^3$H]-methyllycaconitine in the presence of increasing concentrations of either methyllycaconitine (MLA) or α-bungarotoxin. After 120 min, the assay was terminated by aspiration of the buffer and rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester. Filters were washed with approximately 4 x 1ml washes of ice cold assay buffer, and filter disks transferred to scintillation vials containing 5 ml Ecolume scintillation cocktail. Radioactivity was counted

using a Beckman 6500 scintillation spectrometer. Specific binding was calculated by subtracting the non-specific binding, defined by 10 $\mu$M MLA.

D. Western analysis for expression of $\alpha$7 protein

Cells stably transfected with the human $\alpha$7 nAChR were harvested from 10-cm plates and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4). Washed cells were resuspended in 50 mM Tris pH 7.4, 1 mM EDTA containing a cocktail of protease inhibitors (Complete™, Boehringer Mannheim, Indianapolis, IN) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 5 min to remove cellular debris, and the supernatant fraction was centrifuged at 100,000 x g for 120 min to pellet the membranes. The membranes were resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1% SDS) containing protease inhibitor cocktail.

For immunoblot analysis, membranes were solubilized in Tris-Glycine SDS Sample Buffer (Novex, San Diego, CA) containing 5% 2-mercaptoethanol and heated at 65°C for 10 min. Solubilized proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hy-Bond ECL, Amersham, Arlington Heights, IL). Blots were rinsed once in PBS, 0.1% Tween-20 (wash buffer), then blocked for 3 h in 5% Carnation non-fat dry milk dissolved in wash buffer (blocking buffer).

The human $\alpha$7 protein was detected with an antibody raised in goat against a human $\alpha$7-specific peptide (Santa Cruz Biotechnology) . The primary antibody was diluted to 0.5 $\mu$g/ml in blocking buffer and incubated with the nitrocellulose membrane for 3 h at room temperature. The membranes were washed three times in wash buffer. The secondary antibody was peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) diluted 1:2500 in blocking buffer and incubated with membranes for 45 min at room temperature, followed by five changes of wash buffer. The antibody signal was visualized using the ECL developing system (Amersham) according to the manufacturer's directions.

E. Northern analysis for expression of $\alpha$7 encoding message.
Total RNA was isolated from approximately 1 x $10^7$ cells for northern hybridization analysis. Total RNA was size-fractionated on an agarose-formaldehyde gel and blotted to nylon by downward alkaline transfer. Blots were hybridized with digoxygenin-labeled DNA probes specific for human $\alpha$7 subunits (nucleic acid numbers 1066-1533). Blots were hybridized overnight with 20 ng/ml probe and washed at high stringency in a wash buffer containing 0.1X SSPE (3mM NaCl, 0.2mM $NaH_2PO_4$, 0.02mM EDTA) and O.1% SDS at 65°C. Chemiluminescent detection was performed using the Genius 7 kit (Boehringer Mannheim) according to the manufacturer's instructions. Refer to Figure 8.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

Summary of Sequences

Sequence ID No. 1 is a nucleotide sequence encoding an $\alpha_2$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 2 is the amino acid sequence of the $\alpha_2$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 1.

Sequence ID No. 3 is a nucleotide sequence encoding an $\alpha_3$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 4 is the amino acid sequence of the $\alpha_3$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 3.

Sequence ID No. 5 is a nucleotide sequence encoding an $\alpha_4$ subunit of a human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 6 is the amino acid sequence of the $\alpha_4$ subunit of a human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 5.

Sequence ID No. 7 is a nucleotide sequence encoding an $\alpha_5$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 8 is the amino acid sequence of the $\alpha_5$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 7.

Sequence ID No. 9 is a nucleotide sequence encoding an $\alpha_6$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 10 is the amino acid sequence of the $\alpha_6$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 9.

Sequence ID No. 11 is the nucleotide sequence encoding an $\alpha_7$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 7.

Sequence ID No. 12 is the amino acid sequence of the $\alpha_7$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 11.

Sequence ID No. 13 is a nucleotide sequence encoding a $\beta_2$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 14 is the amino acid sequence of the $\beta_2$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 9.

Sequence ID No. 15 is a nucleotide sequence encoding a $\beta_3$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 16 is the amino acid sequence of the $\beta_3$ subunit of human neuronal nicotinic acetylcholine receptor, set forth in Sequence ID No. 15.

Sequence ID No. 17 is a nucleotide sequence encoding a $\beta_4$ subunit of human neuronal nicotinic acetylcholine receptor, and the deduced amino acid sequence thereof.

Sequence ID No. 18 is the amino acid sequence of the $\beta_4$ subunit of human neuronal nicotinic acetylcholine receptor set forth in Sequence ID No. 17.